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CONTENTS.

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CALCIFICATION OF THE BREAST FOLLOWING A TYPHOID ABSCESS.[1]

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Plate I.

The general interest, both pathological and clinical, in the processes of calcification in the animal œconomy, as well as the increasing frequency of the therapeutical use of various salts of calcium, justify a consideration of the following remarkable case.

D. I., a colored house maid, aged 16, was admitted to the Johns Hopkins Hospital on October 2, 1905, on the ninth day of a severe typhoid fever. Her family and personal history were unimportant. Eight days before entry she began to complain of headache and had a chill. On the succeeding day there was abdominal discomfort and occasional "pains in the bones." She felt too ill to work and took some medicine which was followed by diarrhœa—from two to four fluid movements a day—which continued up to the date of entry. There was complete anorexia, and, on several occasions, nausea and vomiting.

On admission the patient was dull and apathetic. The tongue was heavily coated; the pulse, 132; temperature, 101.8°. There were a few coarse râles at the left base. The abdomen was moderately distended and tympanitic. The spleen was not palpable, although the dulness appeared to be increased. Examination of the blood showed: Red blood corpuscles, 3,924,000; colorless corpuscles, 6000; hæmoglobin, 77 per cent. The urine showed a trace of albumin and a positive Diazo reaction. The blood pressure (Riva-Rocci, broad band) was 105. Treatment: milk and albumen water, 120 c.c. alternating every two hours; tub baths, at 85° F., every three hours if the temperature be above 102.5°. On the following day the Widal reaction was positive. The patient soon became very ill. There was considerable tympanities at first which was relieved by turpentine stoops. On October 8 the pulse was slightly irregular; blood pressure (Riva-Rocci, broad band) between 80 and 90. The temperature of the baths was reduced to 75°, and strychnine 0.001 (grs. 1/60) was ordered every four hours, as well as whiskey 15 c.c. (℥ss) p. r. n. The leucocytes varied from 4000 to 7000 to the cubic millimetre.

On October 9, the sixteenth day of the disease, there was a semi-fluid stool with a trace of blood.

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[1] Read before the American Association of Pathologists and Bacteriologists, at Baltimore, 19 May, 1906.

On October 12, the nineteenth day, about 340 c.c. of blood were passed in three stools. The temperature fell from 102.7° in the morning to 99.4° at noon. The pulse rose to 164, the pressure falling at one time to 81.5. At 11 P. M. an infusion of 500 c.c. of 1 per cent. solution of calcium chloride was given under the left breast. The hæmoglobin dropped to 50 per cent. in the evening. The coagulation time was five minutes. An ice bag was placed upon the abdomen; the baths were omitted, and lactate of calcium 2.0 (gr. XXX) every four hours was prescribed.

On the following day, the coagulation time was eleven minutes. Ice sponges were begun again. On October 15, the twenty-second day of the disease, 150 c.c. of blood appeared again in several stools but without any essential change in the condition of the patient. The coagulation time was four minutes on the day preceding the hæmorrhage, and eight and a half minutes on the day following. On October 18, the twenty-fourth day, there was again blood in the stools, for the most part in the shape of old clots, amounting to from 200–800 c.c. by measure. On the 19th, the red blood corpuscles had fallen to 2,200,000; the colorless corpuscles to 3100. From this time on, there was a steady, gradual improvement. On October 23, the thirty-fifth day of the disease, the lactate of calcium was omitted. On the same day, a large abscess was discovered involving the lower half of the left breast, and dissecting around in the interspace just below this as far as the mid-axillary line. There was definite fluctuation just beneath the skin, and harder masses, 1 to 2 cm. in length, could be felt floating free in the pus. The breast was little deformed, the dissection occuring chiefly in the areolar tissue. The point of insertion of the needle with which the infusion of calcium chloride was made fifteen days previously, was not affected, but the abscess was just above this spot, and evidently involved a part of the area in which the infusion was made. The abscess was on the point of rupture and, indeed, did rupture before the operation. Under cocaine, several long incisions were made, two over the abscess proper and one in its extension in the mid-axillary line. A quantity of thin brown pus containing several large sloughs was evacuated. The wound was packed with gauze for drainage.

On October 31, three days later, the wound was irrigated with boric acid and dressed. There was still some sloughing of the actual breast tissues and a prolongation of the abscess up, over and under the mammary gland. On the whole the wound looked fairly clean; it was packed with iodoform gauze. Cultures taken at the time of the opening of the abscess showed Staphylococcus aureus and Bacillus typhosus.

On November 8, eight days later, an examination of the wound, which had been left open with an iodoform dressing, revealed a cartilaginoid induration about the upper part of the opening. There had been an extension around toward the back from the lower part of the drainage incision. On the whole, the wound was clean and of good color. The patient steadily improved, the temperature reaching the normal point on the tenth of November. A light general diet had been allowed for the preceding week.

On November 16, it was noted that the denuded tissue in the wound was slowly changing into a necrotic and almost stony mass. Along the outer side of the breast there was a chain of glands, somewhat swollen and extremely hard. Neither glands nor wound were painful. The suppuration had not extended further toward the back, and the edge of the wound was closing in.

FIG. 1.—Temperature Chart.

On November 20, the following note was made (W. S. T.): "About the opening of the wound there is a perfectly distinct rim of stony hardness. The stony rim of the crater-like opening extends back on the median side about 2 cm. from the edge; on the outer side it is less extensive. The granulations protruding from the outer part of the wound are rather pale, and in them are to be seen whitish streaks and patches that look altogether like the guanin deposits in old ham. These areas feel hard and stony. Passing into the cavity of the wound, the under surface of the protruding granulations corresponds exactly in consistency to that of the outer rim of the crater, the forceps striking similar calcareous areas. In the outer part of the breast, above the opening, and extending along the second interspace are a number of other hard, nodular masses. The largest of these, which is situated a little anteriorly to the mid-axilla, in the third interspace, is as large as a good-sized hickory nut. Above and to the mesial side of this, the nodules are smaller. A number of smaller, indurated areas are to be felt throughout the left breast."

Bits of the white, gritty deposit were removed from the edges of the wound by forceps. These masses were separated easily and with but little bleeding. On burning they emitted a brilliant white light. They dissolved in hydrochloric acid without effervescence. By quantitative analysis[1] it was determined that the calcium, estimated as CaO, comprised 20 per cent. of the dry mass. Curettings of the tissue were examined for tubercle bacilli without result. A guinea-pig, inoculated with scrapings from the edge of the wound, and killed several months later, was free from tuberculosis. Bits of tissue from the wall of the abscess were sent to Dr. Bloodgood on November 14, the following note being made: "The specimens consist of bits of substance that are very firm and seen to be composed of fibrous tissue with a number of small areas of calcification. The section shows granulation tissue on a pretty fibrous basis. In the granulation tissue there are a good many polynuclear leucocytes; lymphoid cells predominate. There are some epithelioid cells. No giant cells or tubercles. Areas of calcification are seen." Further examination showed occasional characteristic giant cells.

The tissue was also examined by Dr. Bunting who made the following note: "The specimen consists of granulation tissue upon a basis of white fibrous tissue. The surface of the granulation tissue shows a fibrin network containing clotted blood, and the immediately underlying portion of the tissue is invaded by polymorphonuclear leucocytes. The granulation tissue is apparently firm and fibrous although, toward the surface, it is extremely cellular, the vessels being surrounded by large numbers of mononuclear cells, both large and small and of the plasma cell variety. Among these one mitotic figure is found. In the deeper layer of the granulation tissue the fibroblasts predominate. In this portion of the tissue there are numerous areas which take a diffuse light bluish stain with hæmatoxylin which on high power seem slightly granular. The tissues in these areas are somewhat swollen and almost hyaline in appearance. The nuclei are pyknotic and the areas are somewhat surrounded and invaded by polymorpho-

[1] The mass was dissolved in hydrochloric acid, the subsequent procedure being that advised by Neubauer and Vogel for the estimation of calcium in the urine. Analyse des Harns, Wiesbaden, 1898, 746.

nuclear leucocytes. In the deeper layers there are numerous multinuclear foreign body giant cells which do not seem to bear any definite relation to the bluish areas described."

The accompanying photograph (Plate I), taken between November 20 and 24, illustrates the condition of the wound.

The course of the breast abscess was slow. On November 24, the skin on both sides had begun to close in but no appreciable change for the better was observable in the denuded breast tissue. A mass half the size of an orange protruded into the wound, both on the inner and outer sides. The mammary gland was still dissected out as previously, the granulation tissue on the surface being transformed, in islands, into a stony yellow material with pale, sluggish granulations between. The calcereous matter at times became loosened and could be picked off in pieces about 2 mm. thick. The hardening of the wound was not so marked as a week before. Along the outer margin of the gland the indurated areas remained as they were two week previously, rounded masses of uniform firmness. In the axilla the glands were somewhat swollen, one being, perhaps, as much as 4 cm. in diameter, but they were soft and did not feel as if calcified. The patient was much better; up and about the ward.

On December 5, the iodoform dressing was removed and the wound was packed loosely with sterile protective rubber strips.

On December 8, three days later, the nodules in the breast appeared to have diminished somewhat in size, while the calcium deposit in the wound seemed less marked.

Acting on the basis of the experiences of Gerhardt and Schlesinger who found that under a carbohydrate free diet, the calcium excretion was increased, the patient was put on the fixed carbohydrate free diet which is described later; this was continued for two weeks.

During this period, the healing of the wound progressed rapidly. On December 18, a note (W. S. T.) states: "Healing continues. The opening of the wound is now not more than 5 cm. in its greatest diameter. The calcified rim is smaller. The masses in the upper and outer quadrant of the breast have diminished in size and hardness. A few nodules are to be felt in the inner part of the breast, but they are very small; it is doubtful whether they have increased in number. Two axillary glands are also somewhat enlarged, the largest, just under the border of the pectoralis, is firm but does not feel stony." Five days later it was again noted (Dr. Thayer) that the calcified rim had softened considerably although there was still a good deal of stony material to be felt. The nodules in the outer part of the breast had diminished in size and firmness. From January 5 to 14, 1906, the patient was again put upon a carbohydrate free diet. By January 9 it was observed that the old calcified rim about the crater-like opening of the wound had wholly lost its stony hardness, having now the consistency of fibrous tissue. One large gland was to be felt in the axilla, while in the breast, over the fourth interspace, there was still a slight suggestion of a firm nodule. Two sinuses running back from the old incisions remained; in the anterior of these, gritty substance could be felt with the probe.

On January 15, there was a slight breaking down at a spot in the scar tissue

below the lower sinus. The wound was packed with iodoform gauze. On the following days there was a distinct deposit of calcareous material to be felt with the probe in the region where it had been in contact with iodoform gauze. On probing the wound the day before, no grating was to be made out. May this, possibly, have been a fresh deposition of calcium in new granulation tissue? Three days later, seventy-one days after the calcification was first observed, the old wound had entirely healed, the hard, sclerotic border having disappeared. The indurated masses in the breast were no longer to be felt. The glands in the axilla had diminished in size. On removing the dressing of the new opening there was a definite induration about the wound, especially in the lower part. No deposits of calcium could be seen or distinctly felt.

On January 21, a small piece of calcium ulcerated through near the lowest sinus. From this time on the sinuses continued to heal steadily. On February 8, but a few small glands were to be felt in the axilla, the largest not so large as the end of the little finger and of no especial firmness. There was no induration to be felt about the mouths of the openings of the sinuses. The patient was discharged, to return to the dispensary for the dressing of the sinuses. On one occasion, during the latter part of the patient's stay in the hospital, after packing a sinus, in which no calcium could be felt, with bismuth gauze, incrustations were found on the following day. It is not impossible, however, that this, as well as the appearance of calcium on January 16, was due to the ulcerating through of deeply seated deposits.

On May 19, the patient returned in response to a letter in order to be exhibited before the Society of Pathologists and Bacteriologists during the reading of this communication. She was perfectly well. The breast showed a puckered scar. There was no induration. The glands in the axilla had almost disappeared.

The results of the estimations by one of us (H. H. H.) of the intake and output of calcium during the several periods above referred to, are of considerable interest. At the outset, on December 3, the patient was put upon a simple diet of eggs and milk, the lime content of which could be easily estimated. It was intended that this diet should be persisted in for a period of a week or ten days but, owing to the strenuous objections of the patient, it was kept up only from December 3 to 8. Estimates of the calcium ingested in the food and excreted in the urine and fæces for three days, December 6 to 8 inclusive, showed a retention of 1.346 gms. or a daily average retention of 0.448.

From December 9 to 23, the patient was given the carbohydrate free diet which is described later. During twelve days, December 11 to 23, inclusive, a study of the intake and output of calcium revealed an excess of calcium elimination over the intake amounting to 2.6229, or a daily excess of calcium elimination amounting to 0.201.

From December 24 to January 6 a regular ward diet was allowed. Owing to the loss of a specimen the record of the calcium metabolism during this period was unfortunately lost.

From January 5 to 14, the patient was again upon a carbohydrate free diet, and during the eight days between February 7 and 14, the excess of calcium elimination over the intake was 1.050 gms., or a daily excess of elimination over intake of 0.131.

From January 27 to February 5, under ordinary ward diet, the intake and output of calcium were nearly equal; there was a retention of calcium amounting to 0.602 or a daily average calcium retention of 0.06 gms.

During the first period of carbohydrate free diet, crystals of calcium oxalate and phosphate were repeatedly found in the urine. During the second period, no calcium crystals were to be found. Diacetic acid and acetone were demonstrated in the urine on January 12 and January 13, but there was no especial increase in Ca elimination on those days.

It is undeniable that, while a diminution in the thickness of the calcified rim of the wound was noted on the day on which the carbohydrate free diet was begun, yet the greater part of the decalcification occurred during the time while the patient was on a carbohydrate free diet. During the two weeks from December 24 to January 6, while the patient was upon a regular diet, but little change was to be made out in the extent of the calcium deposits in the wound.

The association of the excess in Ca elimination with the absorption of the calcareous deposits in the wound and breast is an interesting and striking coincidence. While one may not be justified in assuming that the eliminated calcium represented that which was noted in the breast, it is but natural to suspect that the regime under which the excess in calcium elimination occurred had an influence upon the absorption of the deposits. The elimination of Ca in the urine was, it is true, no greater—indeed not so great—as in Gerhardt and Schlesinger's control case. It must, however, be remembered, in connection with this case, that we were not dealing with a healthy, normal individual, but with one who was convalescing from a long, wasting disease—a condition in which one

might not unreasonably expect to find an appreciable Ca retention. It may, therefore, be true that the excess of Ca eliminated during the periods of carbohydrate free diet, represents a change from the ordinary considerably greater than the figures themselves would suggest. The fact that during each of the intermediate periods there was a Ca retention of a remarkable degree during the first period when the diet was, it is true, of very high lime content, is striking in this connection.

It would be interesting to make further observations as to the intake and output of Ca during, and in the convalescence from, typhoid fever.

The conditions, general and local, under which the deposition of calcium is common are sufficiently familiar. The general conditions: in individuals in whom some process is going on involving the destruction of bone, such as caries, rapidly growing neoplasms and senility, the so-called calcium metastases.

The local conditions: such as caseating areas of tuberculosis in lungs, glands, etc., gummata, areas of regressive change in neoplasms, infarcts, atheromatous areas, old thrombi, the retained fœtus, the incrustation of cartilages and muscles around areas of venous stasis and chronic inflammation, the cardiac valves, pleural and pericardial exudates, hyaline degeneration in various regions, notably in the muscular coats of arteries, in ganglion cells, in the periphery of areas of cerebral softening, in disseminated fat necrosis, equal all conditions in which the affected areas are necrotic, degenerated, or of notably diminished vitality.

Especially interesting are the observations made by Litten who showed that a deposition of lime in the cortex of the kidneys followed temporary ligature of a renal artery in rabbits; a well-marked calcium deposit may be found after an occlusion of two hours' duration. Exactly similar changes have been described by various observers as the result of the ingestion of certain poisons, such as bichloride of mercury (Salkowski), subnitrate of bismuth (Langhans), acetate of lead (Binet), aloin (Gottschalk). Not all conditions, however, favoring necrosis, nor all poisons producing degenerative changes are followed by the deposition of lime salts.

Von Kóssa has recently made a study of the calcification-pro-

ducing poisons. Experimenting with a great variety of substances, he comes to the conclusion that those poisons which especially induce calcification in the rabbit are:

1. The salts of heavy metals (lead, bismuth, mercury and copper).
2. Of the metalloids, iodine and, perhaps, arsenic.
3. Among organic compounds, aloin and iodoform.

In susceptible animals poisoned by these substances the calcium is deposited in the shape of the phosphate; Von Kóssa has never found a deposit of carbonate. Not all the calcium is, however, present in the form of the phosphate; a part is apparently bound in some organic compound.

Inasmuch as the calcium content of the blood and bones of the poisoned (aloin) animal remains unchanged, while that of the urine is diminished, it is evident that the kidney fails to excrete the total amount of the calcium circulating in normal quantity, *i. e.*, the deposition is due to a calcium retention. The rabbit is particularly subject to the action of these poisons, owing probably mainly to the fact that its blood has normally an especially high calcium content. Von Kóssa has, further, demonstrated by the following interesting experiments that an increase in the quantity of the calcium in the circulation favors its deposition under the influence of these poisons. To one animal a certain amount of iodoform was administered by the mouth. To another, a smaller amount was given and, in addition, chloride of calcium subcutaneously. In the latter rabbit alone did calcification occur. The experiment was then repeated with three rabbits, one of which received iodoform alone, another an injection of chloride of calcium alone, and a third iodoform by the mouth and chloride of calcium subcutaneously. In neither of the first two rabbits was there any deposition of calcium. In the third there were marked changes. *In animals in which the calcium content of the blood has been artificially increased it is, therefore, possible to bring about calcification with doses of the poison essentially smaller than those required under ordinary circumstances.*

If one consider the course of events in the case which has just been described, a remarkable analogy appears between the conditions existing here and the experiments of von Kóssa.

1. In a patient suffering from hæmorrhage from the bowel in typhoid fever there were administered, in the course of eleven days, 132 grammes of lactate of calcium by mouth and five grammes of chloride of calcium subcutaneously.

2. In the area in which the subcutaneous injection of chloride of calcium was given there developed an extensive abscess from which Bacillus typhosus and Staphylococcus aureus were obtained.

3. After opening and draining the abscess was packed with iodoform gauze.

4. Extensive areas of calcification developed in the granulation tissue wherever it was exposed to the action of iodoform as well as in other adjacent parts of the breast.

5. It is possible that a similar deposition of calcium followed the dressing of subsequently developed sinuses with subnitrate of bismuth.[8]

In other words, in an individual in whom the calcium content of the organism was probably increased as a result of the administration by mouth and hypodermically of lactate and chloride of calcium, an abscess developed which was dressed with iodoform gauze; this was followed by the deposition of calcium in the granulation tissue immediately adjacent to a substance (iodoform) which is known, experimentally, to favor the deposition of calcium in rabbits. This deposit of calcium disappeared rapidly and completely under a carbohydrate free diet. During three weeks of this period the average daily excess of calcium elimination over that of calcium intake amounted to 0.174 + gr.

We have been unable to find a similar case in literature. Interesting and suggestive in this connection are the observations of Schujeninow who, with Chiari, demonstrated the deposition of calcium in muscle fibres about the borders of laparotomy wounds in seventeen out of twenty-four cases. In the earliest case examined, calcification was discovered thirteen hours after the incision. This reached its height from about the ninth to the twentieth day and then disappeared by absorption. It was noted that the calcification

[8] Although the sequence of events here was remarkable, we are rather inclined to regard the appearance of the calcium after the beginning of the bismuth dressings as due to the breaking through of deeper lying calcium masses.

was always near the ligature and occurred in fibres which had undergone colloid degeneration following the disturbances of circulation as a result of the ligatures. It would be interesting to know whether, in these cases, the wounds were dressed with iodoform. In his experiments with rabbits the wounds were closed with iodoform collodion.

As to the cause of this extensive deposition of calcium our observations do not justify a definite conclusion. One can only point to the remarkable combination of circumstances—the extensive calcification of the granulation tissue and of circumscribed areas throughout the breast after an acute abscess developing at the seat of an injection of calcium chloride in an individual who had been taking, for eleven days, large doses of the lactate of calcium—and further to the fact that the calcification did not appear at the outset, but only after the wound had been packed with gauze impregnated with iodoform, a substance which, experimentally, has been found in some animals to favor the deposition of calcium.

We have endeavored, in several instances, to reproduce this picture in rabbits by administering lactate of calcium subcutaneously, after which an abscess was to be produced, opened and packed with iodoform gauze.

Five rabbits in all were used. Daily injections of 0.13 per 500 grammes weight were administered for ten days. In attempting to produce an abscess, the following method was evolved. Aleuronat meal in from seven to ten times its weight of water to which 3 per cent. starch was added, was injected under the skin. Failing to produce an abscess in this manner, dead cultures of Bacillus typhosus were used with fatal results. One abscess occurred spontaneously and one was obtained by the hypodermic injection of croton oil in ether. Although both were packed with iodoform gauze, in neither case was there a deposit of calcium. No trace of calcium was found on necropsy in kidneys or liver.

Determinations of the intake and output of calcium.—These analyses were made by one of us (Dr. Hazen)[4] in the clinical laboratory of the John Hopkins Hospital under the direction of Dr. C. P. Emerson, according to the following method:

[4] I desire here to express my indebtedness to Dr. Emerson and Dr. Boggs for their assistance in this work. H. H. H.

At the outset the patient was given a teaspoonful of charcoal by mouth. Beginning with the meal following this, a quantity of food exactly similar to that given by mouth was set aside for analysis. The first stool after the charcoal had appeared in the fæces was saved; the urine was collected as soon as the charcoal had appeared. To close the period, charcoal was again administered, after which no food was kept. After the appearance of this charcoal in the fæces, neither urine nor stools were saved.

Great care was taken to insure that equal amounts of the same food were saved for examination; this was personally watched. The food was accurately weighed and dried in a thermostat. The total food for the period was then repeatedly run through a sausage grinder, and when thoroughly mixed, samples were removed for the determination of the Ca. This was done by burning the food until a perfect ash was secured; it was then dissolved in HCl, and the same procedure was followed as in the case of the urine. As it was naturally impossible to get an exact half-portion of the food in the case of the eggs, the following method was adopted. One dozen eggs were analyzed and the mean Ca content used as a basis for estimation. It was found necessary to boil the eggs hard before burning them.

All food analyses were made in triplicate, and in no instance was there the slightest variation in the Ca determination, good evidence that a uniform mixture was secured.

In the case of the stools the same method was employed as for the food, except that they were analyzed in daily quantities. Analyses were made in duplicate.

The urine was saved in twenty-four hour amounts, preserved with chloroform and estimated separately. At first the estimation was made according to the well-known gravimetric method described in the tenth edition of Neubauer and Vogel, p. 746. It was, however, soon found that, in about 15 per cent. of the cases, the Ca was not precipitated, necessitating the use of another specimen. The following modification was accordingly devised which by careful duplicate, comparative tests with the older method, proved entirely reliable, while giving invariably a precipitate of oxalate of calcium.

Two hundred cubic centimetres of the urine were employed. The urine was then acidified with HCl and filtered; NH_4OH was added until a precipitate formed. This suspension was neutralized with acetic acid instead of HCl. An excess of ammonium oxalate and a few c.c. of sodium acetate were then added, and the amount put in an evaporating dish. This was covered with a glass plate and heated over night on a water bath. It was then filtered through an ashless filter paper, until the filtrate was perfectly clear. The precipitate was washed with hot water and burned with the paper in a blast flame, in a platinum crucible until it lost no more weight. This converted the oxalate into the oxide, in which form it was gravimetrically determined. Tests were made in duplicate.

During an initial period in order to determine what she was doing upon a more or less normal mixture the patient was put upon a milk and egg diet. The diet consisted of 375 c.c. of milk and two eggs every four hours. As the patient objected strenuously to its

continuance it was abandoned after three days. In the light of further work it would seem that this diet was unfortunately chosen, as the Ca content of milk is so high that, in the large quantities administered, it was probably partly responsible for the appreciable Ca retention which was demonstrated. The results of the estimates of the intake and output of calcium during this period are shown in the record of Period I in the table. As has been already noted, there was a considerable Ca retention.

Table Showing the Results of Estimates of the Intake and Output of Calcium.

	Ca in Urine.	Ca in Stools.	Total Ca Excretion.	Ca in Food.	Ca Retention.	Ca Excess Elimination.	Daily Average Amount Ca in Urine.	Per Cent. of Ca Excreted in Urine.	Relation of Ca in Urine to that in Stools.	Daily Average Excretion of Ca.	Daily Average Amount of Ca in Food.	Daily Average Retention of Ca.	Daily Average Excretion of Ca in Excess.	Per Cent. of Ca Excreted in Stools.
Period I. 6/xii–8/xii, Inclusive. Milk and Eggs Diet.	.615	5.340	5.955	7.301	1.346	—	0.205	10.32%	11.5:100	1.985	2.433	.448	—	89 67%
Period II. 11/xii–23/xii, Carbohydrate Free Diet.	3 094	4.561	7.655	5.033	—	2.622	0.238	40.4 %	65.6:100	0 588	0.387	—	0.201	59 58%
Period III. 7/i–14/i, Carbohydrate Free Diet.	1.572	4 485	6.057	5.007	—	1.050	0.196	25.95%	35:100	0 751	0.6258	—	0.131	74.04%
Period IV. 27/i–5/ii, Normal Mixed Diet.	1.260	9.849	11.109	11.711	0.602	—	.126	11.34%	12.7:100	1.1109	1.1711	0.06	—	88.65%

From December 11 to 23 the patient was put upon a carbohydrate free diet, essentially the test diet advised by v. Noorden in diabetes.[*] An exact half-portion of every meal was measured and set aside for analysis. The record of Period II on the table shows a reversal of the picture in Period I—a high per cent. of Ca elimination in the urine and a distinct excess in elimination of Ca over the intake. During this period crystals of oxalate and phosphate of lime were repeatedly found in the urine. Acetone and diacetic acid were not demonstrable.

From December 24 to January 6 inclusive, the patient was on ward diet, but, unfortunately, the Ca was not determined, owing to loss of a specimen.

[*] Breakfast: 5 grm. (1/6 oz.) tea steeped in 200 c.c. (6⅔ oz.) water; 150 grm. (5 oz.) ham; one egg.

Lunch: 200 grm. (6⅔ oz.) cold beef; 60 grm. (2 oz.) fresh cucumber (or good quantity of lettuce) with 5 c.c. (teaspoonful) vinegar; 10 c.c. (2

From January 7 to 14 inclusive, the patient was again upon a carbohydrate free regime. During this period no crystals of oxalate of calcium were found in the urinary sediment.

On January 12 and 13, acetone and diacetic acid were found in the urine. There was, however, no increase in Ca elimination in the urine on those days. The record of Period III in the table shows again an excess in the Ca eliminated, though not so marked as in the previous period.

From January 27 to February 5, a study of the calcium intake and output was made, while the patient was on ordinary ward diet. Here, again (Table, Period IV), a slight Ca retention is demonstrable with a marked diminution in the per cent. of Ca eliminated by the urine.

A study of the table shows that the excess of calcium was excreted by the urine, the per cent. of calcium excretion in the stools falling strikingly during the period while the patient was upon a carbohydrate free diet.

It is unfortunate that the relations between the ammonia and calcium output should not have been determined. In the observations of Gerhardt and Schlesinger the ammonia output was found to run parallel with that of the calcium.

The patient remained in excellent health throughout the experiments. She gained materially in weight during the whole period. The weight was taken regularly during the experiment, but owing to an unfortunate misunderstanding, the entries were not regularly made upon her temperature chart. There was a slight loss of not more than a pound or two during each of the periods of carbohydrate free diet, with considerable gains during the intermissions. Thus, on November 26, the weight was 82½ pounds. On December 3, the day that the milk and egg diet was begun, the weight was 86½ pounds. Two weeks later, December 17, in the middle of the first carbohydrate free period, the weight was 84 pounds. A month later, on January 14, at the end of the second carbohydrate

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teaspoonfuls) olive oil and salt and pepper to taste; 20 c.c. (4 teaspoonfuls) brandy with 400 c.c. (13–14 oz.) Apollinaris water (soda water or ordinary water will do); 60 c.c. (2 oz.) of coffee without milk or sugar.

Dinner: 200 c.c. (6⅔ oz.) clear bouillon; 250 grm. (8⅓ oz.) beef (weighed raw) basted with 10 grm. (⅓ oz.) butter; 8 grm. (2⅔ oz.) green salad with 10 c.c. (2 teaspoonfuls) vinegar and 20 c.c. (4 teaspoonfuls) olive oil or 3 tablespoonfuls of some good, well cooked vegetable such as spinage; 3 sardines; 20 c.c. (4 teaspoonfuls) brandy with 400 c.c. (13–14 oz.) Apollinaris water (ordinary water or soda water will do).

Supper: 2 eggs, raw or cooked; 400 c.c. (13–14 oz.) seltzer water (or any good water).

free period, the weight was 103 pounds. On February 8, the day of discharge, the weight was 114 pounds.

* * *

SUMMARY.

1. In a patient with typhoid fever, who had taken, during eleven days, 132 grammes of lactate of calcium by the mouth, as well as 5 grammes of chloride of calcium subcutaneously, there developed, eleven days after the subcutaneous infusion, a large abscess of the breast from which Bacillus typhosus and Staphylococcus aureus were obtained on culture. The abscess was opened and the wound packed with iodoform gauze. Eleven days later a deposit of calcium appeared in the granulation tissue bordering the wound. This rapidly increased until the rim of the opening and the lining granulation tissue were converted into a hard, calcareous mass. A number of nodules of an apparently similar character appeared in other parts of the breast.

2. A month later the iodoform packing was removed and the patient was put on a carbohydrate free diet. This was continued for twenty-four of the next thirty-six days. Under this treatment the abscess almost entirely healed with complete disappearance of the deposit of calcium.

3. Studies of the intake and output of calcium showed that, during three days under a diet of milk and eggs there was a material calcium retention (1.346 grammes): (*b*) That during two periods amounting to three weeks in all, under a carbohydrate free diet, there was an excess of Ca elimination over the intake of 3.672 grammes. (*c*) That during ten days following the last carbohydrate free period, under a normal diet, there was a retention of calcium amounting to 0.602 gramme.

4. The disappearance of calcium from the breast was associated in time with the carbohydrate free diet and the excessive elimination of calcium.

5. Under the carbohydrate free diet acetone and diacetic acid appeared on two occasions in the urine, but were not accompanied by any increase in the elimination of calcium.

6. With regard to the cause of the calcification, we cannot speak with certainty. The remarkable association of the deposition of

calcium at the seat of an injection of calcium chloride in an individual who had been receiving large quantities of lactate of calcium by the mouth—a deposition occurring in tissues which were in direct connection with or in the immediate neighborhood of a dressing containing iodoform, a poison which in certain animals produces degenerative changes in the kidneys and liver which are peculiarly prone to calcification, is striking and worthy of note.

7. In how far the decalcification of the affected area may have been due to the carbohydrate free regime with the consequent excess in calcium elimination, it is impossible, on the basis of a single case, to say. The coincidence in time of the disappearance of calcium from the breast with this treatment is, however, remarkable and extremely suggestive.

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TOXINS AND ANTITOXINS—SNAKE VENOMS AND ANTIVENINS.

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In this article we shall give an account of some quantitative determinations of the relation between the different snake venoms and their specific antivenins. The venoms employed were those of the cobra (*Naja tripudians*), of *Crotalus adamanteus* and of *Ancistrodon piscivorus*. Dr. George Lamb of India has kindly furnished us with cobra venom. The other two were personally procured by one of us (N.). The cobra antivenin was obtained through the kindness of Professor Calmette of Lille. The other two antivenins were prepared at this Institute.

McFarland[1] was the first to attempt the preparation of crotalus antivenin. In this, it appears, he had considerable difficulty, because the animal he used, the horse, reacted to subcutaneous injection with widespread œdema and inflammation. By the use of intravenous injection of unaltered venom McFarland obtained an antivenin which, according to his account, was protective against neuro-toxin, but had scarcely any effect on the irritative principle.

In order to avoid the inherent difficulty of immunization with unmodified venom, Flexner and Noguchi[2] attempted to eliminate the local effects by the use of a dilute solution of hydrochloric acid and of trichloride of iodine. They obtained in this way, in experiments on dogs and rabbits, an antivenin which neutralized perfectly the toxic effects of all the elements of crotalus venom.

In this Institute a *goat* was used, which was injected with unmodified crotalus venom subcutaneously. The animal stood the injection well; œdema was slight, and had disappeared at the end of a few days after each injection. The general condition of the

[1] McFarland, *Proc. of the Soc. of Amer. Bact.*, Dec., 1900.

[2] Flexner and Noguchi, *Jour. Med. Research*, 1903, vi, 363.

animal was apparently unchanged, and its weight slowly increased. (See Table I.)

It will be seen from reference to the protocol of the experiment that the highest antitoxic power was found on March 23 (see Table II) when one cubic centimeter of the serum neutralized the effects of about six minimal lethal doses. A little later the amount of the antitoxin diminished in spite of injections of relatively large doses of the venom. The antitoxin used in the following experiments was obtained on March 30 and 31, 1904.

On May 11, 1904, two kids were born and the amount of antitoxin in the colostrum was determined. The quantity of antitoxin was about the same both in the blood and in the milk. This fact is of considerable interest inasmuch as previous experiments[3] had shown that the amount of antitoxin in the milk was much less than that in the blood. The milk of non-immunized goats was also examined as controls and was found to be devoid of antivenomous action.

Experiments on immunization against the venom of the *water moccasin* were first undertaken with *unmodified* venom injected *subcutaneously*. The goats appeared to stand the injections of small amounts very well, but in two cases (Tables III and IV) the animals died after the injection of 0.2 gram. They had previously tolerated repeated injection of smaller quantities.

To determine whether 0.2 gram was the m. l. d. for goats of this size, 0.15 gram of moccasin venom was injected subcutaneously into a normal goat. But, as shown in the protocol of the experiment (Table V), this animal died after a single subcutaneous injection of 0.15 gram. Then we used the moccasin venom *modified* by placing 0.5 gram of the dried venom, dissolved in one hundred cubic centimeters of one per cent. hydrochloric acid, in the thermostat at 37° C. for twenty-four hours. The solution was neutralized with sodium hydroxide. Immunization with this modified venom was rapidly and easily accomplished (Table VI). A trial serum, drawn on May 13, 1904, showed a weak antivenomous power, but after continued injections of the venom the antitoxic

[3] Salomonson and Madsen, *Ann. de l'Inst. Pasteur*, 1897, xi, 315, and 1899, xiii, 262.

standard rose to a point at which, on May 30, 1904, one cubic centimeter of the serum completely neutralized all effects of 0.0024 gram (2 m. l. d.) of the unmodified venom (Table VII). This antivenin was used in the experiments which are to be discussed later.

Solutions of the venoms were made as follows:

0.5 grm.	dried crotalus	venom	in	100 c.c.	water,
0.5 grm.	dried moccasin	venom	in	100 c.c.	water,
0.4 grm.	dried cobra	venom	in	100 c.c.	water.

These solutions preserved under toluol at a temperature of from 2° to 3° C. were used for all the experiments which follow. The two first solutions showed no perceptible deterioration during the month of experimentation, but the toxicity of the cobra venom was sensibly diminished.

CROTALUS VENOM.

The determination of toxicity was generally made by intraperitoneal injections into guinea-pigs. Table VIII, presenting a small series of experiments, shows in detail the relation between the weight of the animal and the toxicity of the venom. Apparently this relation remains about the same per kilo for animals of from 250 to 500 grams, while smaller animals weighing about 125 grams are much more sensitive. Crotalus venom *passed through a Chamberland filter always loses more than fifty per cent. of its toxicity,* as shown in Table IX. The toxicity is considerably lower and the action is much more irregular when the venom is injected *subcutaneously* (Table X). White rats are very resistant to its action (Table XI). Tables XII and XIII give the details of experiments dealing with the *neutralization of crotalus venom by its specific antivenin.*

To 0.006 gram of venom were added the amounts of antivenin shown in the first column of Table XIII. The mixtures were kept two hours at 37° C. and then injected in the fractional quantities indicated in the second column of the table. Taking the dose which kills after from fifteen to seventeen hours as a unit, we have derived the facts shown in Table XIV, where under "*n*" are indi-

cated the quantities of antivenin added to a constant dose of venom, 0.006 gram, and under "q observed," the observed toxicity.

These observed values, after allowing for errors in experiment, can be expressed by the same formula, which represents the combinations of toxins and antitoxins of other substances.[4]

$$\frac{\text{Free toxin}}{\text{vol.}} \cdot \frac{\text{Free antitoxin}}{\text{vol.}} = K \cdot \text{Toxin-antitoxin.}$$

In this case:

$$\frac{1}{q_0}\left[n\frac{1}{q}\cdot p - \left(\frac{1}{q}-\frac{1}{q_0}\right)\right] = K\left(\frac{1}{q}-\frac{1}{q_0}\right)^2$$

where $1/q$ represents the quantity of antitoxin equivalent to an amount of toxin used and K the constant of association.

With $p = 1$ and $K = 0.0048$, we found the value of q, shown under "q calc. I." For greater convenience these results are graphically represented in Fig. 1. The tracing shows the calculated values of "q," while the dotted circle shows the observed values. It will be noted, therefore, that the values derived from the experiments mentioned above can be expressed according to the simple formula:

$$-\frac{\delta q}{dn} = Kq.$$

We have shown that this formula represents the action of saponin and cholesterin[5]; the column under "q calc. II" shows the correspondence.

The experiments on *rabbits* (Table XV) showed a marked difference between subcutaneous and intravenous injections of venom. For the *neutralization experiments* shown in Table XVI, intravenous injections only were used. The technique was the same as that used in the experiments with guinea-pigs, the quantity of venom being always 0.006 grm. The results obtained by taking as a unit of toxicity a dose killing after five days are summarized in Table XVII. They are shown in Fig. 1 by a dotted square. The form of the tracing obtained agrees to a certain extent with that

[4] Festkrift ved Indvielsen af Statens Serum Institut, Copenhagen, 1902.
[5] Extr. du Bul. de l'Acad. Royale de Danemark, 1904.

determined for guinea-pigs; further calculations were omitted, since the first part of the determination showed an error.[6]

Antivenin produced by immunization has also the power of

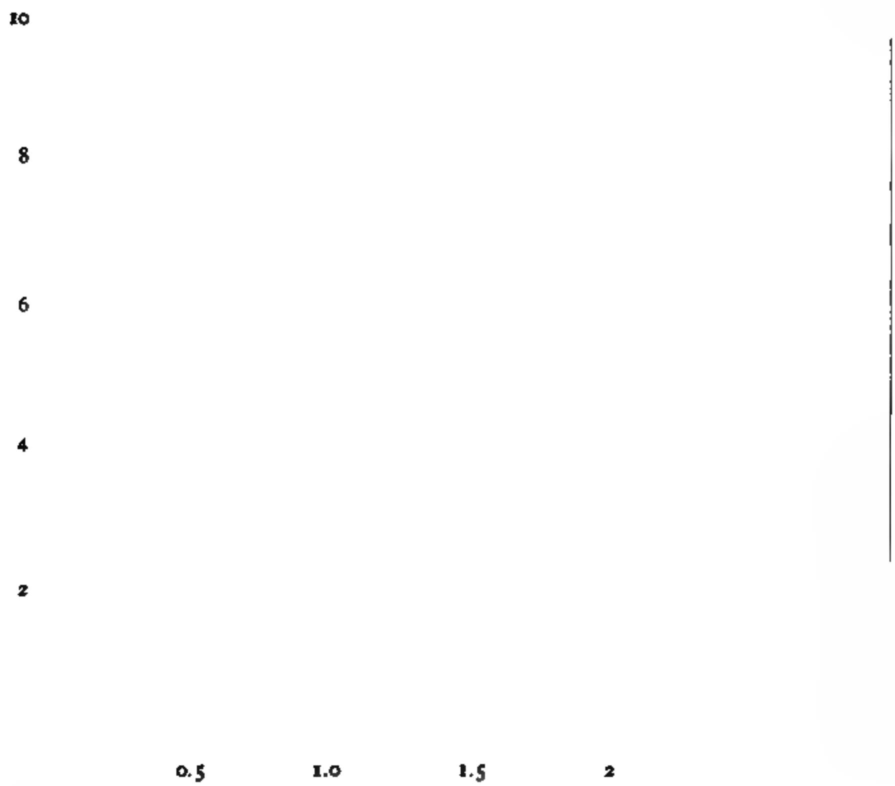

FIG. 1. Crotalus venom-antivenin.

neutralizing the hæmolytic property of crotalus venom. Table XVIII gives the details of experiments; one cubic centimeter of

[6] Perhaps these errors are due to the different methods of injection. At the time when these experiments were carried out, the researches of Morgenroth (*Berliner klin. Woch.*, 1904, xli, 526) on the slow combination of toxin and antitoxin of diphtheria had not been published. Nevertheless, it does not seem unlikely that the reaction will be finished after two hours at 37° C.

0.05 per cent. crotalus venom mixed with the quantities of crotalus antivenin indicated under "*n*" was added to 0.9 per cent. sodium chloride solution to bring the total volume to two cubic centimeters. These mixtures were kept two hours at 37° C. and then their

0.1 0.2 0.3 0.4 0.5

FIG. 2. Crotalus lysin-antilysin (dog's blood).

hæmolytic action, on a five per cent. suspension of dog's blood in 0.9 per cent. saline solution, was examined by the method used in this Institute. In Table XVIII, columns marked "I, II, III," show the doses producing equal hæmolysis; the averages, representing the hæmolytic power, have been estimated from these figures and are given under "*q* obs." As shown graphically in Fig. 2, the form of tracing obtained by neutralization is, in this case, practically a straight line.[1]

[1] The constant of equilibrium was, in this case, almost zero. In the table are shown, under "q calc.," the values estimated on the assumption that 1 c.c. of

COBRA VENOM.

The first determinations of the m. l. d. were made by subcutaneous injections of 0.2 per cent. solution of venom into *guinea-pigs* weighing from 650 to 670 grams. (Table XIX.) A little later another solution of venom, 0.4 per cent., filtered through a Chamberland filter (Table XX) was examined. A comparison was, however, somewhat difficult, because the animals of the two series were not

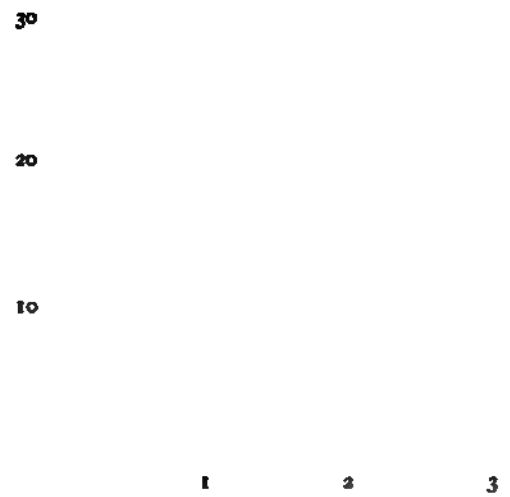

FIG. 3. Toxicity of cobra venom.

of equal weight; it was seen, nevertheless, that filtration did not perceptibly diminish the toxicity of the solution.

The relation between the dose and the toxicity is shown by the tables. In the first place, the time of death is greatly shortened up to a certain point as the dosage is increased. With 0.0005 gram it is 3.75 hours, but beyond this point an increase of the dose does

this antivenin is equivalent to 1.86 c.c. of the solution of crotalus venom. Moreover, for 0.55 c.c. of antivenin there was a discrepancy, pointing to a feeble dissociation of the venom-antivenin combination, corresponding practically to the value of "K" (the constant of dissociation) of 0.0006.

not proportionally shorten the time intervening between the injection and the death of the animal.

In Table XXI are recorded, for the greater part, the same experiments shown in Tables XIX and XX, undertaken, however, with a little larger animals (marked *). If it be arbitrarily assumed, as in a previous paper,[8] that 0.0005 gram is equal to one m. l. d., the results will group themselves with a certain regularity about this dose. Perhaps this will appear more clear by reference to Fig. 3, where the m. l. d. is drawn on the axis of the abscissa, while the time, in hours, is constructed on the ordinates. It will be seen that the determinations form a very regular curve of an asymptotical type. The decrease in time does not agree with the increase in dose; this decrease is perhaps due to the time of incubation and to the method of injection, since it is well known that venom requires some time for absorption from subcutaneous tissues. With intravenous injection the decrease would probably have been much more pronounced. It is evident that the values obtained with doses ranging from 0.6 to 1.2 m. l. d. give the most decisive determinations. The lower and very much larger doses show results which are much less certain. The method just described has, moreover, the advantage that it is not always necessary to find out the dose which will kill within a fixed time. After determining the scale once one can find by interpolation which fraction of the minimal lethal dose is present in a certain mixture.

On account of the very important rôle which lecithin plays in the hæmolysis caused by cobra venom, some experiments were undertaken in order to learn whether one cubic centimeter of a 1/50 *N* solution of lecithin would produce any appreciable change in the toxicity for guinea-pigs. As shown by Table XXII, no such change occurs.

The combination of cobra venom and its specific antivenin seems to occur at 36° C. very rapidly. The small series of experiments, shown in Table XXIII, does not indicate any appreciable difference in the neutralizing power, whether venom and antivenin are mixed together and injected immediately or injected at the end of six

[8] Arrhenius and Madsen, *Acad. Roy. des Sciences et des Let. de Danemark*, 1904.

hours.[9] The mixtures of toxin and antitoxin were incubated for two hours at 36° C.

During the experiments undertaken to explain the neutralization of venom with antivenin, it appeared that the first preparations of antivenin which were at our disposal were too feeble. In Table XXIV will be found in the second column, under "*n*," the quantities of serum (Antivenin I), in cubic centimeters, which were added

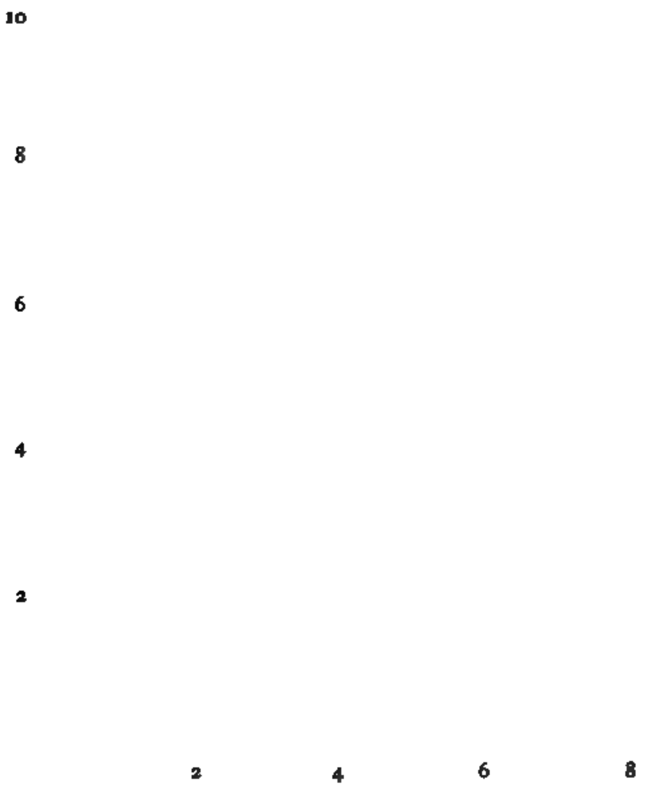

FIG. 4. Cobra venom-antivenin (guinea pigs).

to 0.008 gram (16 m. l. d.), and to 0.004 gram respectively of the venom. In the following column is shown the fraction of this

[9] The experiments were not complete. Experiments with intravenous injection were also lacking, and would be necessary to make it perfectly clear that the reaction between venom and antivenin is rapid (cf. the researches of Morgenroth on diphtheria antitoxin, *loc. cit.*)

mixture which was injected; and in the last two columns the results. One experiment with a new antivenin (II) showed that from ten to twelve cubic centimeters would neutralize the effect of 0.004 gram. It was necessary to know that this antivenomous effect was not due in part to the normal serum of the horse. In one ex-

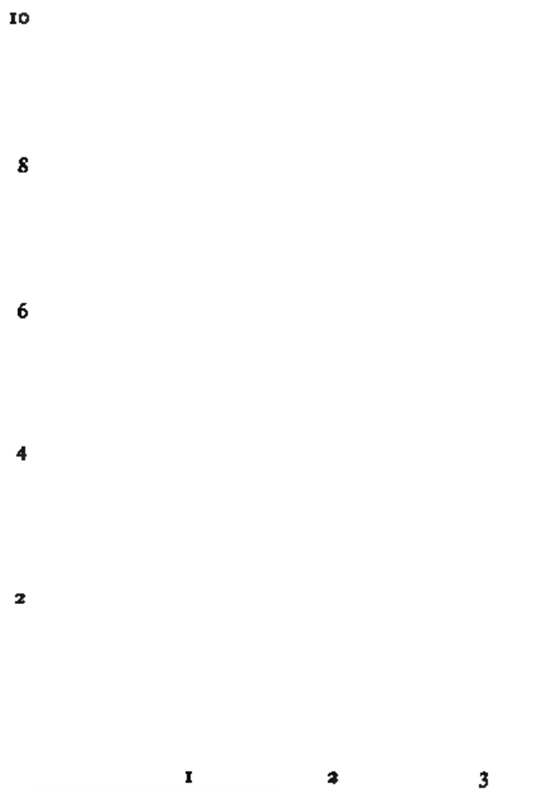

FIG. 5. Cobra venom-antivenin (guinea pigs).

periment twenty cubic centimeters of normal horse serum gave almost no antagonistic effect when tested against 0.008 gram of the venom (Table XXV). With this antivenin (II) (Table XXVI) some experiments upon partial saturation were undertaken. To 0.0028 gram of the venom were added the amounts of antivenin indicated under "*n*." The mixtures were, as usual, incubated

for two hours at 36° C.; then the fractions indicated in the following column were injected intraperitoneally into guinea-pigs. There were series of experiments, one with animals of 370 grams, another with those of 450 grams. The determinations of toxicity, *q*, in the last series (B) will be found in Table XX. They can be summarized as follows:

n	*q*
0	10
2	5
4	3.5
6	2
8	1.7
10	<1

Fig. 4 furnishes a graphical representation of these results.

In one series (guinea-pigs weighing 370 grams) there was no determination made for $n = 0$. The other values are marked by a dotted square in Fig. 4. It will be seen that this curve corresponds very well to the first one.

The last series of *neutralization* experiments were carried out in June, 1904, with an antivenin considerably stronger than that previously used (Table XXVII); four cubic centimeters of this antivenin neutralized effectively 0.003 gram of venom. The results of these experiments may be summarized as follows:

n	*q*
0	11
1	7
2	4
3	1.5 (?)
4	<1

The tracing in Fig. 5, which graphically represents the results of neutralization, is not very strongly curved. The results of neutralization of the *hæmolysin of cobra venom* by its antivenin was tested twice with Antitoxins I and II. The procedure was that which we have usually employed: one cubic centimeter of a 0.1 per cent. solution of cobra venom was mixed with various quantities of antivenin; to these mixtures was further added a sufficient amount of physiological salt solution to bring the total volume to two cubic centimeters. A mixture, kept for two hours at 37°, was

measured into tubes and eight cubic centimeters of a one per cent. suspension of horse's blood corpuscles were rapidly added (each liter of the suspension contained eight cubic centimeters of 1/100N lecithin solution). The tubes were incubated for two hours at 37° C. and then over night at a lower temperature; hæmolysis was estimated by our usual method.

The figures in Table XXVIII are the averages, derived in the

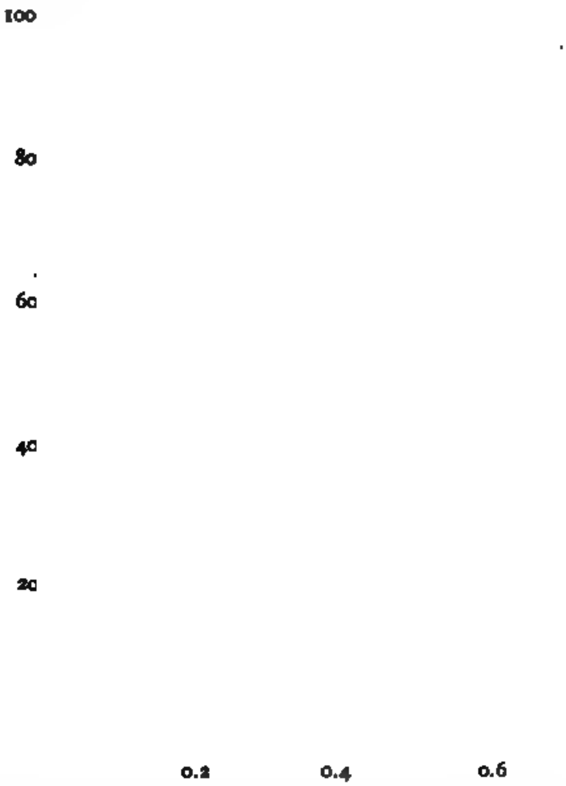

FIG. 6. Cobra lysin-antilysin.

same way as those of the experiments recorded in Table XVIII. In Fig. 6 the observations with Antivenin I are marked by a dotted circle, and with Antivenin II by a dotted square. The tracings, as may be seen, are rather close to straight lines.

The theoretical values of "q calc." are calculated under the sup-

position that one cubic centimeter of Antivenin I is equivalent to 1.98 c.c. of venom solution, and of Antivenin II equivalent to 1.7 c.c. of venom ($K = 0$). For both series, the last values for "q obs." are then considerably lower than the calculated ones. This indicates that there is a clear dissociation[10] (corresponding to $K = 0.0016$). Thus it is not quite correct to assume that the curve of neutralization of cobrolysin and of cobra antilysin is a completely straight line.[11]

WATER MOCCASIN VENOM.

The toxicity of water moccasin venom was examined on two occasions, May 6 and August 22, 1904; during the interval of one hundred and eight days the venom was preserved at from 2° to 4° C. The animals employed for these experiments were guinea-pigs weighing 260 grams. The venom was injected into the peritoneal cavity. A comparison between Tables XXIX and XXX shows that the toxicity was intact. The experiments upon the *neutralization of this venom by its specific antivenin* show singular results. They were carried out by mixing 0.012 gram of venom,—about 10 m. l. d.—with varying amounts of antivenin. After the mixture had been kept for three hours at 37° C. its toxicity was tested by injecting various fractions into guinea-pigs weighing 250 grams. (Table XXXI.) By the addition of 2 c.c. of serum the toxicity was lowered from 10 to 6; of 4 c.c., to about 4 or 5; of 5 c.c., to 3; and of 6 c.c., to about 2.5. But by the addition of still larger amounts of antivenin no further reduction of the toxicity could be produced, as experiments with 8, 9, 10, 20 and 40 c.c. of antivenin showed. This phenomenon was perhaps due to the fact that the antivenin in large enough doses had in itself a toxic action.[12] Unfortunately, our stock serum was exhausted, and we were unable to carry further an investigation of this phenomenon.

Finally, a study was made of the *hæmolytic* action of the venom of the water moccasin; one cubic centimeter of this venom (stock

[10] Myers, *Jour. Path. and Bact.*, 1900, vi, 415. Flexner and Noguchi, *Jour. Path. and Bact.*, 1903, viii, 379.

[11] Kyes, *Berl. klin. Woch.*, 1904, xli, 494.

[12] In fact, it is well known that the normal serum of the goat is often toxic for quinea-pigs.

solution) was mixed with different quantities of moccasin antiserum. The mixtures were incubated for two hours at 37° C. Special experiments had shown that the reaction was complete at the end of ten minutes. The hæmolytic tests were made in the usual way, using a five per cent. suspension of dog's blood. The results are given in Table XXXII and in Fig. 7. It will be seen

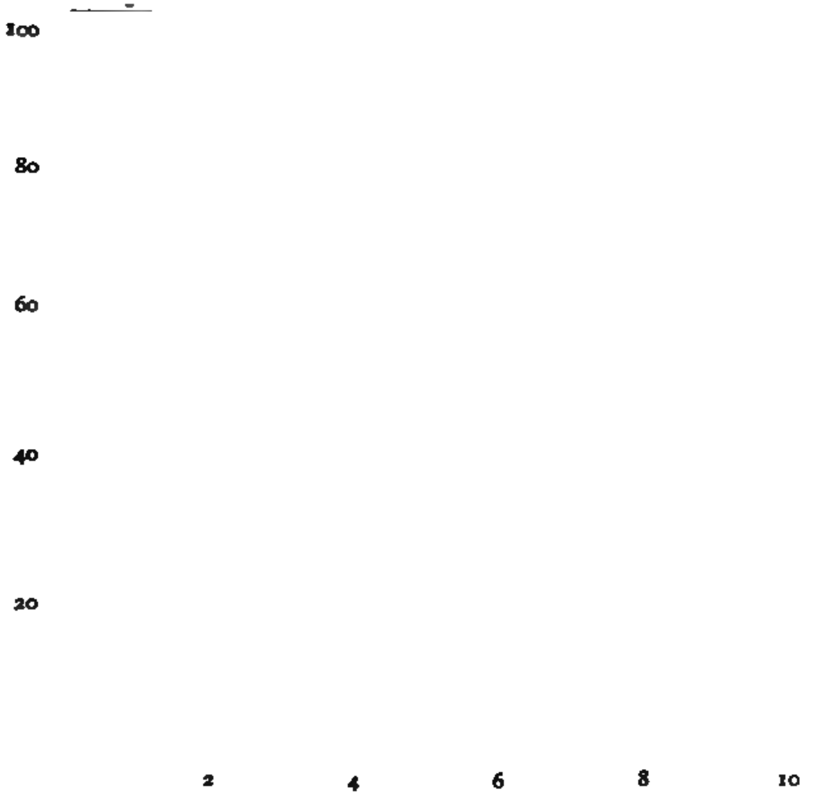

FIG. 7. *Ancistrodon piscivorus* lysin-antilysin.

that the tracing representing the combination is approximately a straight line, except for high concentrations of antitoxin. The values are estimated on the supposition that one cubic centimeter of antivenin is equivalent to 1.2 cubic centimeters of the venom solution, and that $K = 0$. In this case the values calculated for higher concentrations show more variation than in the case of crotalus and cobra lysins. K should be about 0.006.

Unfortunately, the lack of material prevented the continuation of our experiments. Those given are to be regarded as preliminary on account of their small number. For this reason we have not presented any general conclusions, but have given our experiments in the hope that they may prove the starting point of similar researches in the future.

SUMMARY.

A specific antivenin against crotalus venom can be prepared bv the immunization of goats. A specific antivenin against water moccasin venom can be produced by the immunization of goats with this venom, modified by hydrochloric acid. Immunization with the unmodified venom is very difficult.

The toxicity of crotalus venom is diminished more than fifty per cent. by passage through a Chamberland filter. There is a simple relation between the toxicity and the body weight for guinea-pigs weighing from 250 to 500 grams. Smaller guinea-pigs (125 grams) are comparatively less resistant. The toxicity is smaller by subcutaneous than by intraperitoneal injection (guinea-pigs), or by intravenous injection (rabbits). White rats are very resistant.

The toxicity of cobra venom is not measurably diminished by filtration through a Chamberland filter. The relation between the amount of venom and the corresponding time of death is very regular, and can be expressed by a curve of asymptotic nature. Lecithin does not increase the toxicity.

The tracings representing toxin-antitoxin neutralization for the three venoms (crotalus, cobra and moccasin) show deviation from the straight line. This deviation is most pronounced for the toxic quota of the venoms. The tracing representing crotalus venom-antivenin neutralization, determined on guinea-pigs, can, within errors of experiment, be expressed by the equation:

$$\text{Free toxin} \cdot \text{Free antitoxin} = K \cdot \text{toxin-antitoxin}.$$

The corresponding tracing determined on rabbits is somewhat different, but both tracings are much more markedly curved than that for cobra venom-antivenin. The neutralization tracing of water moccasin venom shows the peculiarity, that small amounts of anti-

TABLE I

IMMUNIZATION OF A GOAT WITH CROTALUS VENOM.

Nov.	4,	1903,	0.0001 grm.	
	7		0.0002	
	10		0.0004	
	13		0.0008	
	16		0.0012	
	20		0.0016	
	23		0.002	
	26		0.0025	
	30		0.003	
Dec.	4		0.0035	
	9		0.004	
	12		0.004	
	16		0.005	
	21		0.008	
	26		0.01	
	31		0.015	
Jan.	2,	1904,	0.02	
	6		0.032	
	13		0.04	
	16		0.04	
	19		0.05	
	23		0.08	
	28		0.1	
Feb.	2		0.14	
	10		0.16	
	15		0.2	Slightly ill.
	23		0.05	Blood was drawn before the injection (1 c.c. of this serum protected against 2.5 m. l. d.).
	29		0.1	
Mar.	4		0.2	Slight swelling around the site of injection.
	9		0.25	
	15		0.3	
	19		0.35	
	23		0.4	Bled (1 c.c. protected against 6 m. l. d.).
	30			Bled about 350 c.c. (1 c.c. protected against 5 m. l. d.).
	31			Bled about 500 c.c. (1 c.c. protected against 5 m. l. d.).
Apr.	18		0.05	Bled (1 c.c. protected against 0.4 m. l. d.).
	20		0.1	
	24		0.15	
	27		0.2	
	30		0.35	
May	6			Bled (1 c.c. protected against 4 m. l. d.).
	7			Bled (1 c.c. protected against 3.2 m. l. d.).
	11			Two kids born. The milk was tested and it was found that 1 c.c. protected against 2.4 m. l. d.

venin decrease the toxicity to a minimum, but the toxicity is again increased by further addition of antitoxin.

The tracing representing neutralization of the hæmolysins of the three venoms are different from the tracings of neutralization of the toxins, and approach very closely to a straight line. Still, in all instances, the determinations with great concentrations of antilysin show pronounced deviation, perhaps due to some dissociation of the toxin-antitoxin combination.

TABLE II.

TESTS OF THE ANTIVENOMOUS POWER DURING IMMUNIZATION.

The animal was bled on February 23, 1904. Guinea-pigs of 500 grm. were used for the experiment.

Serum (antivenin) 2 c.c. + crotalus venom 0.002 grm. = No symptoms.
Serum (antivenin) 2 c.c. + crotalus venom 0.004 grm. = Sick for 3 days.
Serum (antivenin) 2 c.c. + crotalus venom 0.005 grm. = Sick for 2 days; recovered.
Serum (antivenin) 2 c.c. + crotalus venom 0.0075 grm. = Death in 27^h 7^m.
Serum (antivenin) 2 c.c. + crotalus venom 0.01 grm. = Death in 5^h 45^m.

The animal was bled on March 23, 1904. Guinea-pigs of 400 grm. were used.

Serum 2 c.c. + crotalus venom 0.0075 grm. = No symptoms.
Serum 2 c.c. + crotalus venom 0.01 grm. = Sick for 1 day; recovered.
Serum 2 c.c. + crotalus venom 0.0125 grm. = Death in 3^h 35^m.

The animal was bled on March 30 and 31, 1904. Guinea-pigs of 280 grm. were used.

Serum 2.5 c.c. + crotalus venom 0.005 grm. = No symptoms.
Serum 2.5 c.c. + crotalus venom 0.006 grm. = No symptoms.
Serum 2.5 c.c. + crotalus venom 0.0075 grm. = Death in 12^h.
Serum 2.0 c.c. + crotalus venom 0.006 grm. = Death in 16^h.
Serum 4.0 c.c. + crotalus venom 0.01 grm. = Death in 17^h.

The animal was bled on April 18, 1904. Guinea-pigs of 240 grm. were used.

Serum 2.5 c.c. + crotalus venom 0.0015 grm. = No symptoms.
Serum 2.5 c.c. + crotalus venom 0.003 grm. = Death in 12^h.

The animal was bled on May 6, 1904. Guinea-pigs of 290 grm. were used.

Serum 2.5 c.c. + crotalus venom 0.005 grm. = Slightly ill for 1 day; recovery.
Serum 2.5 c.c. + crotalus venom 0.006 grm. = Death in 12^h.

The animal was bled on May 7, 1904. Guinea-pigs of 290 grm. were used.

Serum 2.5 c.c. + crotalus venom 0.004 grm. = No symptoms.
Serum 2.5 c.c. + crotalus venom 0.005 grm. = Death in 7^h.
Serum 2.5 c.c. + crotalus venom 0.006 grm. = Death in 12^h.

The animal was bled on May 11, 1904. Guinea-pigs of 290 grm. were used.

Serum 2.5 c.c. + crotalus venom 0.003 grm. = Slightly ill; recovery.
Serum 2.5 c.c. + crotalus venom 0.004 grm. = Death in 12^h.

The milk of the same goat was taken several hours after the delivery.

Colostrum 2.5 c.c. + crotalus venom 0.003 grm. = Slightly ill; recovery.

TABLE III.

Immunization of a Goat with the Venom of Water Moccasin by Subcutaneous Injection.

Nov.	7, 1903,	0.0001	grm., no symptoms.
	10	0.0002	
	13	0.0004	
	16	0.0008	
	20	0.0012	
	23	0.0016	
	26	0.002	
	30	0.0025	
Dec.	4	0.003	
	9	0.004	
	12	0.004	
	16	0.005	
	21	0.008	
	26	0.01	
	31	0.015	
Jan.	2, 1904,	0.02	
	6	0.032	Small abscess at the site of injection.
	13	0.04	
	16	0.04	
	19	0.05	
	23	0.08	
	28	0.1	
Feb.	2	0.14	
	10	0.16	
	15	0.2	Ill after 24 hours.
	20		Very ill, but no local symptoms; dyspnœa, loss of appetite and unable to stand up.
	21.		All symptoms above described increasing.
	22.		Died during the foregoing night.

TABLE IV.

Immunization of a Goat with the Venom of Water Moccasin by Subcutaneous Injection.

Feb.	29, 1904,	0.01	grm.
Apr.	4	0.025	No symptoms.
	9	0.04	
	15	0.07	
	19	0.09	
	23	0.12	A large abscess formed at the point of injection.
May	2	0.14	Quite well.
	6	0.18	
	9	0.2	Death after 18 hours.

TABLE V.

TOXICITY OF THE VENOM OF WATER MOCCASIN FOR A GOAT WEIGHING 27 KILO.

On April 10, 1904, at 11:43 A. M. 0.15 grm. (in 5 c.c.) of the venom of water moccasin was injected at the right side of the back, subcutaneously. After from three to four hours the animal became very inactive, but was still able to stand. It avoided lying down or moving. At the same time the whole of the injected side became intensely swollen and very painful. At the end of ten hours after the injection the animal was still able to support its body on the legs. The swelling at the site of injection became more marked and over the whole body there was swelling and œdema in varying degree, but more intense in the region of the injection. The skin around the needle puncture showed no necrosis. Death ensued at the end of twenty-two hours after injection, with symptoms of great dyspnœa.

Autopsy was performed one hour after death. There was no rigor mortis. There was enormous swelling of the entire cadaver, especially pronounced over the abdominal wall and the right back, which was very œdematous. A large quantity of bloody exudate had accumulated in the subcutaneous tissues and the exudate had a jelly-like consistence. The muscles had a dark-purple or deep blackish color, the muscles along the spinal column being darkest. The muscles were friable and very easily torn into pieces. They were infiltrated with extravasated blood corpuscles as well as with free hæmoglobin. The peritoneum, omentum, and serous membranes of the pleuræ and pericardium were free from hæmorrhage. The hæmorrhage in the subcutaneous tissue did not extend into the cervical region. Two dead fœtuses found in the uterus presented marked rigor mortis, but almost no hæmorrhage.

TABLE VI.

IMMUNIZATION WITH THE VENOM OF WATER MOCCASIN MODIFIED BY HYDROCHLORIC ACID.

Dried venom 0.5 grm.; 1 per cent. aqueous solution of hydrochloric acid 100 c.c. The mixture was kept 24 hours at 37° C.

After incubation for 24 hours at 37° C., the mixture was neutralized with hydrate of sodium, which produced a bulky precipitate of the proteids of the venom. A goat was injected subcutaneously.

Apr.	13, 1904,	0.025 grm.	
	18	0.025	
	20	0.04	
	22	0.05	
	24	0.07	
	26	0.1	
	28	0.2	
	30	0.2	
May	2	0.3	
	4	0.4	
	6	0.7	
	13		Bled.
	14	0.4	
	18	0.2	
	21	0.3	
	24	0.5	
	30		Bled (600 c.c.).

TABLE VII.

Tests for Antivenin during Immunization.

Serum was drawn on May 13, 1904. Guinea-pigs of 290 grm. were used.
Serum 2 c.c. + moccasin venom 0.0036 grm. = Death in 7^h and 20^m.
Serum 2 c.c. + moccasin venom 0.006 grm. = Death in 2^h.
Serum 2 c.c. + moccasin venom 0.008 grm. = Death in 1^h and 50^m.
Serum 2 c.c. + moccasin venom 0.012 grm. = Death in 40^m.

Serum was drawn on May 30, 1904. Guinea-pigs of 400 grm. were used.
Serum 2 c.c. + moccasin venom 0.012 grm. = Death in 36^h.
Serum 2 c.c. + moccasin venom 0.012 grm. = Death in 1^h and 30^m.
Serum 2.5 c.c. + moccasin venom 0.006 grm. = Recovered.
Serum 2.5 c.c. + moccasin venom 0.008 grm. = Death in 10^h.
Serum 2.5 c.c. + moccasin venom 0.01 grm. = Death in 4^h 10^m.
Serum 2.5 c.c. + moccasin venom 0.012 grm. = Death in 3^h 40^m.

TABLE VIII.

Relation between the Body Weight and Susceptibility of Guinea-pigs after Intraperitoneal Injection of Crotalus Venom (not Filtered).

Guinea Pigs of 500 grm.		Guinea Pigs of 250 grm.		Guinea Pigs of 125 grm.	
Venom in grm.	Result.	Venom in grm.	Result.	Venom in grm.	Result.
0.0012	+ 9^h 25^m	0.0006	+ 10^h 10^m	0.0003	+ 9^h 5^m
0.001	+ 18^h	0.0005	+ 18^h	0.00025	+ 6^h
0.0008	+ 12^h 25^m	0.0004	§ very ill	0.0002	+ 12^h
0.0008	§	0.0004	§	0.0002	+ 5^h 30^m
0.0008	§	0.0003	§	0.00015	+ 9^h
0.0008	§			0.0001	§ sick
				0.00008	§

+ = Death. § = Recovery.

TABLE IX.

Determination of the Minimal Lethal Dose of Crotalus Venom Filtered Trough the Chamberland Bougie.

The stock solution was made as follows: Dried venom, 0.5 grm. and distilled water, 100 c.c. Guinea-pigs weighing 250 grm. were used and the venom was administered intraperitoneally on April 5, 1904.

Venom in grm.	Result.	Remarks.
0.0002	§	Almost no symptoms; the abdominal tension was slightly increased for one day.
0.00025	§	Ditto.
0.0003	§	Ditto.
0.00035	§	Sick for one day; the abdominal tension was much increased the first day.
0.0004	§	Ditto.
0.0005	§	Very sick for the first day; the abdominal tension was enormously increased during two days.
0.0006	§	Ditto.
0.001	§	Very sick for two days; had a very high abdominal tension; after 3 days improved rapidly.
0.0012	+ 15^h	
0.0015	+ 10^h	

+ = Death. § = Recovery.

TABLE X.

DETERMINATION OF THE MINIMAL LETHAL DOSE OF CROTALUS VENOM WITHOUT FILTRATION.

The stock solution was made as follows: Dried venom 0.5 grm.; distilled water 100 c.c. Guinea-pigs of 320 grm. were injected subcutaneously, April 21–23, 1904.

Venom in grm.	Result.	Remarks.
0.0035	+ 14 days	First day, very sick; second day, the site of injection highly swollen and the hairs came off about the site of needle puncture; the skin was softened and a small amount of a sero-sanguineous fluid discharged from the needle puncture, but the animal seemed more active than on the first day. The appetite was normal. The ulcerated area of the skin assumed greyish color (necrotic) on the third day. The animal was perfectly active until 12 days after injection, when it showed progressive emaciation, while locally there was a solid infiltrated scar. Death occurred with symptoms of marasmus.
0.005	+ 4^h	Three hours later the animal showed grave general symptoms and collapsed. Locally there was intense swelling of the tissues without any actual ulceration.
0.005	+ $3^h 35^m$	Two hours after the injection the animal became seriously ill. Marked hæmorrhagic œdema around the site of injection, which finally spread over the entire injected side.
0.0075	§	The animal showed only slight general symptoms, while an extensive ulcer formed about the point of injection on the second day, but became dry after 7 days.
0.01	§	Showed slight stupefaction during the first day; on the second day an ulcer (3 x 4 c.c.) formed around the site of injection, but dried after 7 days.
0.015	+ $1^h 25^m$	One hour after injection the animal fell; the local swelling was very marked. At autopsy there was very marked hæmorrhage in the muscular layers, but only moderate hæmorrhage in the mesentery.

+ = Death. § = Recovery.

TABLE XI.

TOXICITY OF CROTALUS VENOM FOR RATS (200 GRM.) AFTER INTRAPERITONEAL INJECTION.

Venom in grm.	Result.
0.0002	Well.
0.0005	Well.
0.001	Well.
0.0015	Abdominal tension increased; next day well.
0.0016	Well.
0.0018	Well.
0.002	Well.
0.002	Well.
0.002	Death 4^h.
0.0022	Well.
0.003	Well.

TABLE XII.

Determination of the Minimal Lethal Dose of Crotalus Venom (not Filtered).

Guinea-pigs weighing 270 grm. were injected intraperitoneally, April 6 to 16, 1904.

Venom in grm.	Result.	Remarks.
0.00035	§	Sick for 2 days; local infiltration disappeared after 7 days.
0.0004	§	Very sick for 2 days; local infiltration marked. 9 days later the local infiltration disappeared; after 11 days the animal was quite normal.
0.00045	+ 13 days	First day, critically ill; second day, some improvement in condition, but the whole abdominal wall was softened and formed an ulcer, 3 x 3 cm., from which a sero-sanguineous fluid discharged; third day, the surface of the ulcerated area was becoming dry and the appetite was usual; fourth day, the ulcer presented a darkish hue and the edge was demarkated. After a week the ulcer dried with a blackish crust, which gradually became smaller and fell off. The animal was nevertheless much emaciated and finally died.
0.0005	$+ 17^{h}$	Animal immediately became highly irritable and uneasy after 15 to 30 minutes; abdominal tension steadily increased. None of animals showed appetite after the injection and the hairs were ruffled. In one hour the abdominal tension became very high, and the wall became a purplish color, due to hæmorrhage. A sero-sanguineous fluid was discharged from the needle puncture. The animals became weaker gradually and finally succumbed. At autopsy there was very marked hæmorrhage in the peritoneum and viscera, and in the muscular layers of the abdominal wall.
0.00055	$+ 17^{h}$	
0.0006	$+ 15^{h}$	
0.0006	$+ 21^{h}$	
0.0006	$+ 19^{h}$	
0.001	$+ 3^{h} 48^{m}$	
0.001	$+ 4^{h} 34^{m}$	

+ = Death.
§ = Recovery.

TABLE XIII.

CROTALUS VENOM AND ANTIVENIN (I) INJECTED INTRAPERITONEALLY.

Guinea-pigs weighing 250 grm. were used. April 15, 1904. The venom was

Venom 0.06 gr. + n c.c. Serum.	Divided by.	Result.	Symptoms of the Animals which Finally Recovered.
0.25	8	+ 10^{h}	
	9	+ 15^{h}	
	10	§	Sick for 2 days; slight infiltration around the site of injection, lasting for 5 days.
0.5	6	+ 10^{h}	
	7	+ 15^{h}	
	8	§	Slightly ill for 1 day; slight infiltration around the needle puncture lasting for 4 days.
0.75	4	+ 12^{h}	
	5	+ 23^{h}	
	6	§	Slightly ill for 1 day.
1.0	3	+ 10^{h}	
	3½	+ 17^{h} 20^{m}	
	4	§	Slightly ill for 1 day; slight infiltration lasting for 2 days.
	5	§	Almost no symptoms.
	6	§	Ill for several hours only.
1.25	2½	+ 15^{h}	Sick for 2 days; slight local infiltration lasting for 2 days.
	3	§	
1.5	1½	+ 7^{h}	Sick for 3 days; was very ill the first day. Local infiltration lasted for 3 days.
	2	§	
1.75	1	+ 7^{h}	
	1½	+ 15^{h}	Sick for 3 days; marked local swelling, lasting for 3 days.
	2	§	
2.0	1	+ 15^{h}	
	1½	§	Sick for 2 days; local infiltration for 3 days.
2.25	½	+ 17^{h}	
	½	+ 12^{h}	Very sick for 2 days; local infiltration marked and lasted for 3 days; recovery in 4 days.
	1	§	
2.5	½	§	Slightly ill for one day; recovery in 2 days.
	1	§	Almost no symptoms.
	1	§	No symptoms.
	1	§	No symptoms.

+ = Death.
§ = Recovery.

TABLE XIV.

CROTALUS VENOM-ANTIVENIN INJECTED INTO GUINEA-PIGS.

n	q Obs.	q Calc. I.	q Calc. II.
0	12	11.5	11.8
0.25	9	9	8.7
0.5	7	7	6.4
0.75	4.5	5.2	4.7
1	3.5	3.7	3.5
1.25	2.5	2.5	2.55
1.5	1.7	1.8	1.9
1.75	1.5	1.25	1.4
2	1	0.87	1.1
2.25	0.5	0.5	0.55
$p=1.$	$K=0.048.$	$K=0.053.$	$n=3/2.$

TABLE XV.

TOXICITY OF CROTALUS VENOM UPON RABBIT.

1. *Subcutaneous Injection.* (*Left Inguinal Region.*)

Venom in grm.		Weight of Animal.	Result.
0.01	25/iv	2000 grm.	+ 7 days
0.015	26/iv	2100	+ 18^h
0.02	27/iv	2100	+ $10^h\ 25^m$
0.02	16/v	1600	+ $7^h\ 40^m$
0.04	17/v	1700	+ $2^h\ 40^m$

2. *Intravenous Injection.*

0.00025	7/vi	2000 grm.	§
0.0003	7/vi	2000	+ 9 days
0.0003	2/vi	1800	+ 3 days 12^h
0.0003	24/vi	1450	+ 3 days 4^h
0.0004	30/vi	1550	§
0.0004	7/vi	2000	+ 4 days 16^h
0.0004	15/vi	2000	§
0.0005	15/vi	2000	+ 4 days 16^h
0.0005	7/vi	2000	+ 3 days
0.0005	2/vi	1750	+ $17^h\ 50^m$
0.0005	20/v	1600	+ 14^h
0.001	20/v	1600	+ 6–14^h
0.000461	11/vii	1650	§
0.0005	11/vii	1650	+ 5 days.
0.000545	11/vii	1550	+ 4 days 20^h
0.0006	11/vii	1500	+ 4 days

+ = Death.
§ = Recovery.

TABLE XVI.

Crotalus Venom-Antivenin Injected Intravenously.

Rabbits weighing about 1700 grm. were used. July 10 to 20, 1904. The venom was not filtered. Crotalus Antivenin I was used.

Venom 0.006 grm. + *n* c.c. Serum.	Divided by.	Result.
0	10	+ 4 days 12^h
	11	+ 4 days 20^h
	12	+ 5 days
	13	§
0.5	4	+ 4 days 12^h
	5	+ 5 days 7^h
	6	§
	7	§
1.0	2	+ 5 days 7^h
	3	+ 5 days 9^h
	4	§
1.5	1½	+ 5 days 6^h
	2	+ 5 days 9^h
	2½	§
2.0	1½	+ 4 days 12^h
	2	§
	2½	§
2.5	½	+ 4 days 12^h
	1	+ 5 days 20^h
	1½	§
3	1	§

+ = Death. § = Recovery.

TABLE XVII.

Crotalus Venom-Antivenin.

n	*q* Obs.
0	12
0.5	5
1.0	3
1.5	2
2.0	1.5
2.5	1.0

TABLE XVIII.

Crotalolysin.

5 per cent. suspension of dog's blood.
1 c.c. of 0.05 per cent. crotalus venom.
+ *n* c.c. of anticrotalus serum.
+ 1 — *n* c.c. 0.9 per cent sodium chloride solution.

n	I.	II	III.	*q* Obs.	*q* Calc.
0	0.02	0.016	0.006	100	100
0.05	0.023	0.018	0.0065	89	90.5
0.1	0.026	0.02	0.0078	77.4	81
0.15	0.028	0.023	0.0085	70.6	72

n	I.	II.	III.	q Obs.	q Calc.
0.2	0.03	0.025	0.01	63.5	62.5
0.25	0.033	0.029	0.0125	54.4	53
0.3	0.0385	0.035	0.015	45.9	44
0.35	0.052	0.043	0.019	35.8	34.5
0.4	0.078	0.065	0.026	24.4	25
0.45	0.11	0.098	0.047	15.8	16
0.5	0.275	0.24	0.12	6.3	6.5
0.55	1.5	1.15	0.35	1.5	0

TABLE XIX.

Determination of the Minimal Lethal Dose of Cobra Venom.

Stock solution was made as follows: Dried venom, 0.1 grm.; distilled water, 50 c.c.; no filtration. Guinea-pigs weighing 650 grm. were injected subcutaneously February 15, 1904.

Venom in grm.	Result.
0.00005	§
0.0001	+ 23 days
0.00015	§
0.0002	+ 36^h
0.00025	+ $18^h\ 29^m$
0.0003	+ 12^h
0.0005	+ $3^h\ 35^m$
0.00075	+ $2^h\ 3^m$
0.001	+ $3^h\ 34^m$
0.0015	+ $1^h\ 30^m$

+ = Death. § = Recovery.

TABLE XX.

Determination of the Minimal Lethal Dose of Cobra Venom.

Stock solution: Dried venom, 0.4 grm.; distilled water, 100 c.c.; filtered through a Chamberland bougie. Guinea-pigs were injected subcutaneously February 20 to March 2, 1904.

Venom in grm.	Weight of Animals in grm.	Result.
0.0001	530	§
0.00015	470	§
0.0002	550	§
0.0002	480	§
0.00025	450	§
0.0003	460	+ 13^h
0.0004	450	+ $4^h\ 39^m$
0.0005	450	+ $2^h\ 35^m$
0.001	460	+ $1^h\ 59^m$
0.0015	620	+ $1^h\ 42^m$
0.002	600	+ $1^h\ 43^m$

+ = Death. § = Recovery.

TABLE XXI.

RELATION BETWEEN DOSE AND TOXICITY.

Dose in grm.	M. l. d.	Time of Death in Hours.
0.0002	0.4	36
0.00025	0.5	18.5
* "	"	17
0.0003	0.6	12
* 0.0004	0.8	6.16
0.0005	1	3.5
* "	"	4
0.00075	1.5	2
0.001	2	3.5
* "	"	3.67
0.0015	3	1.5
* "	"	1.75
* 0.002	4	1.75

TABLE XXII.

DETERMINATION OF THE MINIMAL LETHAL DOSE OF COBRA VENOM WHEN INJECTED WITH LECITHIN.

Stock solution of cobra venom was made as follows: Dried venom, 0.4 grm.; distilled water 100 c.c.; filtered through Chamberland bougie.

The dose of lecithin injected with the venom was 1 c.c. of 1/50 N. solution. The animals used were guinea-pigs weighing 600 gram. The venom was injected subcutaneously February 24, 1904.

Venom in grm.	Result.
0.0002	§
0.0003	+ 7^h
0.0004	+ $10^h\ 30^m$
0.0005	+ $3^h\ 47^m$

+ = Death.
§ = Recovery.

TABLE XXIII.

VELOCITY OF THE REACTION BETWEEN COBRA VENOM AND CALMETTE'S ANTIVENIN.

The venom-antivenin mixture was injected intraperitoneally into guinea-pigs weighing 600 grm.

Cobra venom 0.008 grm. + Calmette's antivenin 5 c.c.

Simultaneously	1 Hour Contact at 36° C.	6 Hour Contact at 36° C.
+ 90 minutes	+ 85 minutes	+ 85 minutes

TABLE XXIV.

Cobra Venom and Calmette's Antivenin I.

Calmette's Antivenin I. 17/ii/'04.				Calmette's Antivenin I. 17/ii/'04.			
Weight of Guinea Pig in grm.	Venom 0.008 grm. +*x* c.c. Serum.	Divided by	Result.	Weight of Guinea Pig in grm.	Venom 0.004 grm. +*x* c.c. Serum.	Divided by	Remarks.
650	3	4	+ 8 min.	710	3.0	10	$+ 3^h 4^m$
650		7	$+ 3^h 12^m$	670		13½	$+ 10^h 20^m$
650		10	$+ 2^h 8^m$	690		20	$+ 15^h 20^m$
				770		28	$+ 5^h 25^m$
700	4	4	$+ 3^h 2^m$				
700		5	$+ 3^h 33^m$				
690		6	$+ 3^h 36^m$				
550		4	$+ 2^h 35^m$				
540	5	6	$+ 2^h 50^m$				
550		10	$+ 7^h 50^m$				
590		13½	§				

+ = Death.
§ = Recovery.

TABLE XXV.

Cobra Venom and Calmette's Antivenin II.

The venom solution was filtered through the Chamberland bougie before use March 9, 1904.

Calmette's Antivenin II.				
Quantity of the Antivenin Used in c.c.	Cobra Venom in grm.	Weight of Guinea Pig in grm.	Result.	Remarks.
10	0.004	450	$+ 25^m$	
10	0.0036	430	$+ 43^m$	
10	0.0032	480	$+ 5^h 41^m$	
10	0.0028	450	§	No general symptoms; a slight local œdema lasting for 2 days.
10	0.002	440	§	No symptoms.
One bottle (ca. 12).	0.008	510	$+ 26^m$	
"	0.0048	480	$+ 32^m$	
"	0.004	570	§	No general symptoms; marked local œdema lasting for 4 days; no loss in weight.
"	0.0032	490	§	No symptom.
Normal horse serum, 20	0.008	600	$+ 15^m$	
Normal horse serum, 20		600	§	Slightly inactive after 1 hour, but well after 6 hours.

+ = Death.
§ = Recovery.

TABLE XXVI.

Cobra Venom and Calmette's Antivenin. II.

The venom solution was filtered before use. The mixture of venom and antivenin was injected intraperitoneally into guinea-pigs.

Series A. Guinea Pigs of 370 grms.			
Calmette's Antivenin II. 11–12/iii/'04			
Venom 0.0028 grm. + x c.c. Serum.	Divided by.	Result.	Remarks.
2	5	+ 12^h	
	6	+ 26^h	
	7	§	Slightly ill for one day.
	8	§	Almost no symptoms.
4	3	+ 55^m	
	4	+ 36^h	
	5	§	Sick for one day.
	6	§	Almost no symptoms.
6	2.5	+ 60^m	
	3	+ 26^h 9^m	
	4	§	Slightly ill for one day.
8	1.5	+ 28^m	
	2	§	Slightly inactive for several hours.
	2.5	§	No symptoms.
9	1	§	Sick for one day.
10	1	§	No symptom.

Series B. Guinea Pigs of 450 grms.			
Calmette's Antivenin II. 11–12/iii/'04.			
Venom 0.0028 grm. + x c.c. Serum.	Divided by.	Result.	Remarks.
2	4	+ 27^m	
	4	+ 17^h 40^m	
	5	+ 48^h	
	6	§	Almost no symptoms.
4	2.5	+ 1^h 36^m	
	3	+ 14^h 40^m	
	4	§	Sick several hours.
6	2	+ 40^h	
	2.5	§	Sick for two days.
	3	§	Slightly ill for several hours.
8	1	+ 31^m	
	1.5	+ 1^h 6^m	
	2	§	Sick for two days.
	2.5	§	Slightly ill for one day.
10	1	§	Almost no symptoms.

+ = Death.
§ = Recovery.

TABLE XXVII.

Neutralization of Cobra Venom with Calmette's Antivenin.

The venom-antivenin mixture was injected subcutaneously into guinea-pigs weighing 250 grm.

Cobra Venom 0.003 grm. + n c.c. Serum.	Divided by.	Result.
0	8	+ 2^h
	9	+ $3^h\,30^m$
	10	+ $2^h\,30^m$
	11	+ 3^h
	12	+ 36^h
	13	§
	14	§
	15	§
1.0	5	+ 4^h
	6	+ 6^h
	7	+ 8^h
	7	+ $2^h\,30^m$
	8	§
	8	§
2.0	3	+ 5^h
	4	+ $2^h\,30^m$
	4	+ 4^h
	5	§
	5	§
	6	§
3.0	1	+ 36^h
	2	+ 3 days
	2	§
	2.5	§
3.5	1	+ $5^h\,20^m$
	1	+ $3^h\,45^m$
	1.5	§
4	1	§
	1	§

+ = Death. § = Recovery.

TABLE XXVIII.

1 c.c. 0.1 % Cobra Venom. + n c.c. of Antivenin I. + 1–n c.c. of Salt Solution (0.9 %).			1 c.c. 0.1 % Cobra Venom. + n c.c. of Antivenin II. + 1–n c.c. of Salt Solution (0.9 %).		
n	q obs.	q calc.	n	q obs.	q calc.
0	100	100	0	100	100
0.1	78.2	79.5	0.1	83.5	82.5
0.2	56.6	59.5	0.15	71.3	74
0.3	39.8	40.5	0.2	64.5	66
0.4	21.6	20.5	0.25	56.2	57
0.5	5.3	1	0.3	44.4	48.5
			0.4	33.3	32
			0.5	15.9	15
			0.6	3.3	0

TABLE XXIX.

Determination of the Minimal Lethal Dose of Water Moccasin Venom.

Stock solution was made as follows: Dried venom, 1.0 grm.; distilled water, 100 c.c.; the solution was not filtered. The venom was injected intraperitoneally into guinea-pigs May 5, 1904.

Venom in grm.	Weight of Animal in grm.	Result.	Remarks.
0.0005	250	§	Sick for one day.
0.0008	250	§	Ditto.
0.001	250	§	Ditto.
0.001	250	§	Ditto.
0.001	250	§	Ditto.
0.0012	250	$+7^h$	Marked hæmorrhage in the abdominal cavity.
0.0012	250	$+6^h 20^m$	Ditto.
0.0014	250	$+45^m$	Very marked hæmorrhage in the abdominal cavity.
0.0024	420	$+4^h 40^m$	Ditto.
0.0024	420	$+4^h 35^m$	Ditto.
0.0024	420	$+3^h 50^m$	Ditto.
0.0024	420	$+4^h 20^m$	Ditto.

+ = Death.
§ = Recovery.

TABLE XXX.

Determination of the Minimal Lethal Dose of Water Moccasin Venom.

The stock solution of venom was as follows: Dried venom, 1 grm.; distilled water, 100 c.c. The solution which was not filtered was preserved in the ice chest for 108 days. Animals were injected August 22, 1904.

Venom in grm.	Weight of Animal in grm.	Result.
0.0006	250	§
0.0008	250	§
0.001	250	§
0.001	250	§
0.0012	250	$+5^h$
0.0012	250	+6 days
0.0012	250	$+5^h 20^m$
0.0013	250	$+5^h$
0.0014	250	$+4^h$
0.0014	250	$+4^h$
0.0024	420	$+4^h 40^m$
0.0024	420	$+4^h 35^m$
0.0024	420	$+3^h 50^m$
0.0024	420	$+4^h 20^m$

+ = Death.
§ = Recovery.

TABLE XXXI.

Neutralization of Water Moccasin Venom.

The time of contact was 3 hours at 37° C. The animals used were guinea-pigs weighing 250 grm., and the injection was made intraperitoneally.

Venom 0.012 grm. + n c.c. of Serum.	Divided by.	Result.
2	5	+ 9^h
	5	+ 6^h
	6	+ $6^h\ 30^m$
	6	+ $10^h\ 17^m$
	7	§
	7	§
	8	§
	8	§
4	3	+ 3 days
	4	+ $5^h\ 30^m$
	4	+ 16^h
	5	+ $6^h\ 20^m$
	5	§
	6	§
5	3	+ $7^h\ 20^m$
	4	§
	5	§
6	2	+ 5^h
	2.5	+ 16^h and 5^h
	3	§
	3	§
	4	§
8	1.5	+ $6^h\ 30^m$
	2	+ 18^h
	2	+ 16^h
	2.5	+ 22^h
	2.5	§
9	1	+ $3^h\ 10^m$
	2	+ $4^h\ 10^m$
	3	+ 14^h
10	1.5	+ $5^h\ 30^m$
	2	§
	2.5	+ $6^h\ 40^m$
	3	+ $7^h\ 10^m$
20	2	+ 9^h
	3	+ 3^h
	4	+ 3^h
40	4	+ 3^h

TABLE XXXII.

1 c.c. of 0.05 per cent. solution of Ancistrodon piscivorus venom + 2 c.c. of antivenin + 1 — n c.c. of 0.9 per cent sodium chloride solution.

n	q Obs.	q Calc.
0	100	100
0.05	93	94
0.1	87	88
0.15	82	82
0.2	77	76
0.25	70	70
0.3	63	64
0.4	53	52
0.5	42	40
0.6	26	28
0.7	17	16
0.8	10.5	4
1	2	0

EXPERIMENTS ON THE LEUKOLYTIC ACTION OF THE BLOOD SERUM OF CASES OF LEUKÆMIA TREATED WITH X-RAY AND THE INJECTION OF HUMAN LEUKOLYTIC SERUM IN A CASE OF LEUKÆMIA.

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CONTENTS.

Since the discovery of the remarkable effects of X-ray upon the blood, spleen, and glands in leukæmia, numerous attempts have been made to explain its action. Experiments for this purpose have been fruitful, but they have been, for the most part, restricted to animals. Human experiments are obviously preferable to animal ones, whenever they can be carried out, for the reaction of a healthy animal to X-ray is not necessarily the same as that of man in disease. In such an investigation as the present one it is desirable to work with human material, since leukæmia is rarely found in animals, and it has not been produced artificially.

For the puropse of observation we were fortunate in having at our disposal four cases of leukæmia, three of which were of the lymphatic and one of the splenomyelogenous variety. These cases exhibited many phases of reaction to X-ray treatment. One patient gave a favorable therapeutic result; another, a fair result; a third,

a slight improvement only; a fourth, a good result, followed by a relapse.

Finally, we were able to inject the blood serum of the patient who was most improved directly into a patient who was not under the influence of the X-ray.

I. THE EFFECT OF THE BLOOD SERUM OF LEUKÆMIC PATIENTS EXPOSED TO X-RAY UPON THE LEUKOCYTES OF INJECTED ANIMALS.

It was first pointed out by Heineke[1] that lymphoid cells are very susceptible to X-ray. Linser and Helber[2] after numerous animal experiments concluded that X-ray destroys not only the lymphoid cells of the glands and spleen, but also the leukocytes of the circulating blood, attacking first the young mononuclear forms. They found, furthermore, that following the treatment a new substance, a leukotoxin, was produced in the blood, that when injected into animals destroyed the circulating leucocytes, and when added to animal exudates containing leukocytes, caused loss of motion and degeneration of the cells.

A series of animal injections was carried out by us on similar lines to those of Linser and Helber, except that we used human instead of animal serum. The leukocytes of the animals treated were counted before the injection, and at intervals of about two, five, eight, twenty-four, and forty-eight hours afterward. The maximum effect was usually seen in twenty-four hours, but was sometimes delayed until forty-eight hours. The count made at the expiration of the first twenty-four hour period was used for comparison with the normal.

A. *Normal Human Serum.*

Experiment 1.—Blood drawn from the veins of a healthy man (C.) with Luer syringe was defibrinated and centrifuged under aseptic conditions. 3 c.c. of serum injected into rabbits caused in twenty-four hours an increase of about 10 per cent. in the number of leukocytes.

Experiment 2.—5 c.c. of the same serum caused in the same time an increase of 12 per cent. in the number of leukocytes.

Experiment 3.—3 c.c. of serum taken from a healthy man (S.) injected into

[1] Heineke, *Münch. med. Woch.*, 1904, li, 785.

[2] Linser and Helber, *Cong. für innere med.*, 1905, xxii, 143.

rabbits caused in twenty-four hours an increase of 5 per cent. in the number of leukocytes.

Experiment 4.—3 c.c. of the same serum injected into guinea-pigs caused in twenty-four hours a rise of 15 per cent. in the number of leukocytes.

B. *Leukæmic Serum.*

Experiment 5.—The blood was obtained from a case of lymphatic leukæmia (Eich.) before the X-ray treatment was begun. 2.5 c.c. of the serum were injected into a rabbit and caused in twenty-four hours a rise of 25 per cent. in the number of leukocytes.

Experiment 6.—2.5 c.c. of the same serum injected into a guinea-pig was followed by a rise of 5 per cent. in the number of leukocytes.

Experiment 7.—The blood was obtained from a case of splenomyelogenous leukæmia (Butcher). 2.5 per cent. of the serum injected into a rabbit was followed in twenty-four hours by a rise of 50 per cent. in the number of leukocytes.

Experiment 8.—2.5 c.c. of the serum injected into a guinea-pig was followed in twenty-four hours by a rise of 20 per cent. in the number of leukocytes.

Experiment 9.—The blood was obtained from a case of lymphatic leukæmia (Knox) which had not been under treatment for three months. 3 c.c. of the serum injected into a rabbit was followed in twenty-four hours by a rise of 56 per cent. in the number of leukocytes.

Experiment 10.—2.5 c.c. of the same serum injected into a guinea-pig was followed in twenty-four hours by a rise of 20 per cent. in the number of leukocytes.

C. *Leukæmic Serum from a Patient under X-ray Treatment.*

Experiment 13.—The blood was obtained from a case of lymphatic leukæmia (Fifield) which had been under regular X-ray treatment for eighteen months, and which showed, at the time of bleeding, glands and spleen of almost normal size, and a leukocyte count of less than 10,000 per c. mm.

2.5 c.c. of the serum injected into a rabbit was followed in twenty-four hours by a decrease of 35 per cent. in the number of leukocytes.

Experiment 14.—2.5 c.c. of the same serum injected into a guinea-pig was followed in twenty-four hours by a decrease of 60 per cent. in the number of leukocytes.

Experiment 15.—The blood was obtained from a case of lymphatic leukæmia (Knox) under regular X-ray treatment for two months, which had been much improved, but in which the glands and spleen were still enlarged, and the leukocytes varied from 30,000 to 50,000 per c. mm. 3 c.c. of the serum injected into a rabbit was followed by a fall of 40 per cent. in the number of leukocytes.

Experiment 16.—3 c.c. of the same serum injected into a guinea-pig was followed by a decrease of 12 per cent. in the number of leukocytes.

Experiment 17.—The blood was obtained from a case of splenomyelogenous leukemia (Butcher) which had been under regular X-ray treatment for three months. The spleen was about one-half as large as at the beginning of the treatment, and the leukocytes varied from 40,000 to 60,000 per c. mm. 2.5 c.c.

of the serum injected into a rabbit was followed in twenty-four hours by a fall of 20 per cent. in the number of leukocytes.

Experiment 18.—2.5 c.c. of the same serum injected into a guinea-pig was followed in twenty-four hours by a fall of 10 per cent. in the number of leukocytes.

Summary.—The injection of normal human serum in rabbits and guinea-pigs is followed in twenty-four hours by a slight increase in the number of leukocytes.

The injection of serum from cases of leukæmia is followed by a more marked rise in the number of leukocytes.

The injection of serum from cases of leukæmia which have been treated by X-ray causes within twenty-four hours a distinct fall in the number of leukocytes. The fall is greatest with the serum of patients who have shown the most decided improvement under X-ray treatment. The fall is least with the serum of patients who have received the slightest benefit from the treatment. We are, therefore, warranted in concluding that the amount of leukolytic substance present in the blood of these cases probably varies directly with the degree of clinical improvement observed in the cases.

Differential leukocyte counts of the blood of animals injected with the serum showed that the mononuclear cells are more influenced than the polynuclear ones. Hence the leukolytic action is, to a certain extent, selective.

II. THE EFFECT OF X-RAY ON PHAGOCYTOSIS.

In these experiments on phagocytosis the following method was employed: The blood to be tested was defibrinated and placed in two tubes, one of which was exposed to the X-ray for twenty minutes, while the other was kept as a control. To both of these was added a definite amount of a twenty-four hour culture of anthrax bacillus or staphylococcus, after which the tubes were well shaken. At intervals of thirty minutes, one hour, and at intervals of from two to four hours, a drop was taken from each tube, spread on a slide with rice paper and stained with Leischman's stain. A large number of leukocytes, never less than fifty, was then counted, and the percentage of leukocytes containing bacteria was noted. By comparing these results with those observed in the controls one could say whether X-ray treatment increased or diminished phagocytosis.

The blood of two healthy men, and of one case of splenomyelogenous and one of lymphatic leukæmia, was treated in this way. As it was thought that the glass of the test tube might cut off many of the X-rays, most of the experiments were repeated, using a shallow vessel with a cotton covering for the blood. In this case the rays were applied from above. The results were practically identical with the test tube experiments.

In normal blood and in lymphatic leukæmia the X-ray did not materially alter the number of phagocytes or the number of bacteria in the leukocytes. In splenomyelogenous leukæmia, phagocytosis was slightly impaired. These results are easily interpreted when one considers that the polynuclear cells are the only leukocytes capable of phagocytosis, whereas the cells influenced chiefly by the X-ray are the mononuclear ones and the myelocytes, the polynuclear cells being most resistant. In other words, the X-ray may destroy large number of myelocytes or mononuclear cells without materially affecting the phagocytic power of the polynuclear cells.

In the light of these observations it is difficult to understand the assertion of Quadrone[3] that X-ray, by stimulating the phagocytic power of the leukocytes, increases the resistance of animals to disease. He injected various pathogenic bacteria into mice and guinea-pigs, and found that the animals which had been exposed several times to X-ray resisted the infection better than the control animals.

III. THE EFFECT OF THE SERUM OF LEUKÆMIC PATIENTS EXPOSED TO X-RAY ON THE HUMAN LEUKOCYTES AND ERYTHROCYTES, AS OBSERVED IN THE HANGING DROP.

The technique was as follows: The leukocytes used in each experiment were obtained by centrifuging a tube of freshly defibrinated blood; from the top of this tube a platinum loop was filled and placed on a cover-glass. This drop was made up of a large number of leukocytes mixed with a good many red corpuscles. A loop of the serum which was to be tested was added to the drop, and the two were thoroughly mixed on the cover-glass. The cover was placed on a hollow-ground slide and the edges were rimmed with vaselin to prevent drying. The effect of the serum on both the

[3] Quadrone, *Cent. für innere Med.*, 1905, xxvi, 521.

leukocytes and erythrocytes was observed and compared with the controls.

All of the experiments in the table were repeated with leukocytes which had been washed free of red corpuscles and serum with normal salt solution. The behavior of the leukocytes was essentially the same as in the unwashed specimens.

It will be noted that the three cases of leukæmia which were employed represent entirely different clinical reactions to the X-ray treatment. The first (Eich) responded only slightly; the second (Butcher) improved considerably; and the third (Fifield) was improved to the extent of being quite free from subjective or objective symptoms of the disease. Experiments 14 and 15 were intended to show whether the serum of Butcher, which responded only partially to X-ray, had any neutralizing effect upon the strong leukolytic serum of Fifield. The results on this point were not conclusive.

Summary.—1. Leukæmic serum which has not been treated has no leukolytic action on normal blood nor on other leukæmic blood. It has little or no agglutinating action on normal erythrocytes or on the erythrocytes of other leukæmias.

2. The serum of cases of leukæmia which has been treated with X-ray has a decided leukolytic action on normal blood and on other leukæmic blood, and this effect is in direct ratio to the clinical reaction of the patient to the X-ray, as shown by symptomatic improvement.

3. The leukolytic action is selective, showing a marked preference for the mononuclear cells and the myelocytes.

4. The serum of leukæmia which has been treated with X-ray agglutinates the erythrocytes of normal blood and other leukæmic blood. The degree of agglutination corresponds roughly to the degree of leukolysis present.

IV. THE EFFECT OF HEAT ON LEUKOLYTIC BLOOD.

According to Linser and Helber, the leukolytic substance is destroyed by heating the serum to 55° or 60° C. Experiments 9 and 10 with the hanging drop (see Table) and the experiments repeated with the washed leukocytes showed only an impairment of leukolytic action of the serum of leukæmia treated with X-ray after

EXPERIMENTS ON THE EFFECT IN THE HANGING DROP OF SERUM OF X-RAYED LEUKEMIA UPON HUMAN BLOOD.

No. Exp.	Mixture of One Loop Each.		Effect Upon.	
	Leukocytes with Many Red Cells.	Serum.	Red Cells.	Leukocytes.
1	Normal (Coles).	0.9% NaCl sol.	No rouleaux; no clumps.	No swelling in 60°; no fragmentation in 20°.
2	Normal (Coles).	Normal (Thomas).	Rouleaux formation; no clumps.	Swelling in 6°; no fragmentation in 18°.
3	Normal (Thomas).	Normal (Coles).	Moderate rouleaux; no clumps.	Only swelling in [illegible] in 20°.
4	Normal (Thomas).	Lymph. leuk. (Eich)[4] before x-ray.	No tendency to clump.	No swelling in 4[illegible] 20°.
5	Normal (Thomas).	Same after x-ray in vivo.	Moderate degree of clumping.	Swelling in 4°; fragmentation slight in 10°.
6	Normal (Thomas).	Same after x-ray in vivo and also in vitro.	Same degree of clumping.	Swelling in 4°; fragmentation moderate in 10°.
7	Normal (Thomas).	Sp. myel. leuk. (Butcher) x-ray in vivo.[5]	Moderate amount of clumping.	Swelling in 3°; fragmentation in 8°.
8	Normal (Thomas).	Same x-ray in vivo and also in vitro.	Same amount of clumping.	Swelling in 3°; fragmentation more marked in 8°.
9	Normal (Thomas).	Same heated 60° C. for 30′.	Same as above.	Swelling and fragmentation slight; less marked.
10	Normal (Thomas).	Lymph. leuk. (Fifield) x-ray in vivo.[6]	Clumping very marked and immediate.	Swelling and clearing in 2°; fragmentation in 4°, very marked.
11	Normal (Thomas).	Same heated 60° C. for 30′.	Same.	Less marked, but still considerable.
12	Sp. myel. leuk. (Butcher).	Normal (Coles).	No clumps; rouleaux present.	No swelling in 6°; no fragmentation in 20°.
13	Lymph. leuk. (Fifield).	Normal (Coles).	No clumps; rouleaux present.	No swelling in 6°; no swelling in 20°.
14	Normal (Thomas).	1 loop (Butcher) + 1 loop (Fifield).	Clumping marked.	Swelling and fragmentation less marked than X; [illegible] same as VII.
15	Normal (Thomas).	2 loops (Butcher) + 1 loop (Fifield).	Clumping fairly marked.	Swel[illegible] marked than XIV.
16	Lymph. leuk. (Fifield).	Sp. myel. leuk. (Butcher).	Moderate clumping.	Swel[illegible] in 6°, especially [illegible]
17	Sp. myel. leuk. (Butcher).	Lymph. leuk. (Fifield).	Clumping very marked and immediate.	Swel[illegible] in 3–4°, espe-[illegible]
18	Sp. myel. leuk. (Butcher).	Same heated 60° C. for 30′.	Same.	Considerably less swelling and fragmentation.

[4] Eich—lymphatic leukæmia; only slight reaction to X-ray; leukocytes = 300,000.

[5] Butcher—splenomyelogenous leukæmia; considerable improvement under X-ray; leukocytes = 40,000.

[6] Fifield—lymphatic leukæmia; very marked improvement under X-ray; leukocytes = 8,000.

being heated for thirty minutes to 60° C. The agglutinating effect of the serum upon the erythrocytes was not altered by the heat. However, we consider these experiments inconclusive as to whether the leukolytic substance is thermolabile or thermostabile. For, if the leukolytic substance is made up of amboceptor and complement, and the complement is destroyed by heat, fresh complement is supplied by the test leukocytes in the hanging drop. The same objection exists to injecting the serum, after heating, into animals, for fresh complement may be furnished by the blood of the animals.

We attempted to obtain anticomplement, which, by replacing the destroyed complement, would render the leukolysin inert; but the animal injected for this purpose died during the process of immunization.

V. THE EFFECT OF X-RAY UPON THE BLOOD OF LEUKÆMIC PATIENTS IN VITRO.

A. *Normal Blood.*

Experiment 1.—2 c.c. of normal defibrinated blood (S.) were exposed for twenty minutes to X-ray in a test tube, at a distance of four inches. Under the microscope, no swelling nor fragmentation of the leukocytes were seen. When a loop of serum was added to a loop of normal leukocytes (C.) and examined in the hanging drop, within ten hours no leukolysis was observed.

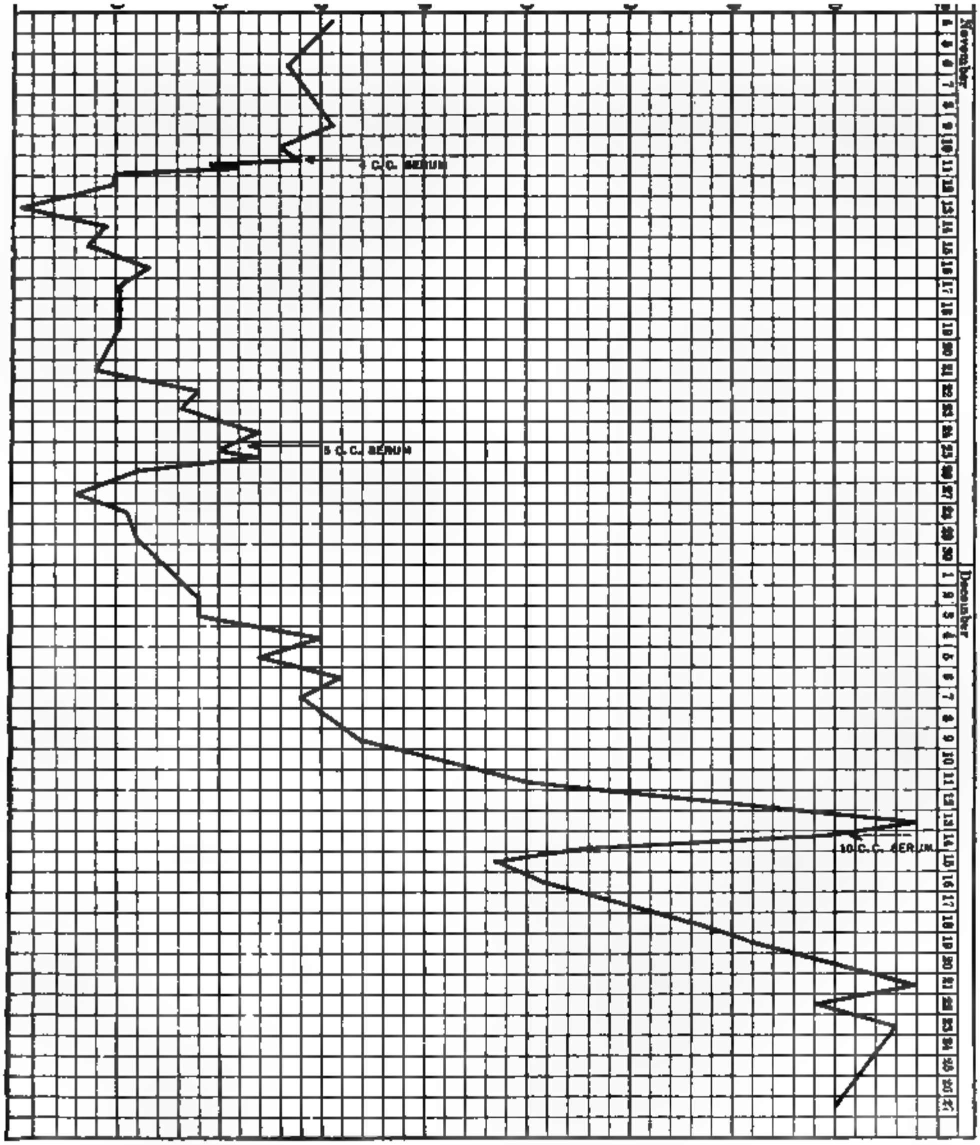
Experiment 2.—The above experiment was repeated, using normal blood (C.) for the X-ray exposure, and normal leukocytes (S.) for the test leukocytes. The results were identical.

B. *Leukæmic Blood.*

Experiment 3.—In a case of lymphatic leukæmia (Eich.) which had been treated four times with X-ray, the serum of which is slightly leukolytic, the leukocytes numbered about 300,000. After a thirtv-minute exposure in a test tube to X-ray, the leukolytic action of the serum was increased, and the leukocytes showed, in the next twelve hours, more swelling and fragmentation than those in the control tubes.

Experiment 4.—A case of splenomyelogenous leukæmia (Butcher) which was under X-ray treatment most of the time for twelve months, showed only moderate reaction. The leukocytes numbered about 50,000 and the serum was moderately leukolytic. After X-ray treatment for thirty minutes, in the course of the next ten hours the leukocytes gave evidence of greater disintegration than the control leukocytes. The myelocytes were the most affected, and the serum became more leukolytic.

Experiment 5.—A case of lymphatic leukæmia (Knox) at first improved under the X-ray treatment and then relapsed. The leukocytes numbered about


November
December
5 C.C. SERUM
10 C.C. SERUM

40,000. Before treatment, the serum was not leukolytic, and after being treated in a tube for thirty minutes, it was not leukolytic to any extent. The leukocytes in the exposed tube showed a slight swelling and an earlier fragmentation than those in the control tube.

Summary.—Single direct exposure to X-ray of normal blood in the test tube causes no appreciable destruction of the leukocytes, nor is the serum leukolytic to any extent. Direct exposure of leukæmic blood produces varying degrees of fragmentation of the treated leukocytes and imparts, in some cases, a leukolytic property to the serum. The development of leukolytic substance seems to be greatest when the leukocytes are most abundant, and slight or absent when but few leukocytes are present. It is probable that in normal blood too few leukocytes are present to produce any considerable amount of leukolysin.

VI. THE EFFECT OF X-RAY UPON THE BLOOD OF LEUKÆMIC PATIENTS IN VIVO.

Single Exposure.

Experiment 1.—A patient with splenomyelogenous leukæmia (Butcher) had taken no X-ray treatment for twelve weeks. The leukocytes numbered about 36,100. After vigorous treatment with X-ray for thirty minutes over the spleen and liver, three hours later, the leukocytes numbered about 29,800; six hours later, they numbered 26,000; twelve hours later, 28,000; thirty hours later, 26,000. A differential count made it evident that the decrease had been chiefly in the myelocytes. Before the X-ray treatment, about 5 per cent. of the leukocytes were fragmented; twenty-four hours after treatment nearly 20 per cent. were fragmented.

Experiment 2.—A case of lymphatic leukæmia (Knox) had been given no X-ray treatment for over a month. The glands of the neck and spleen were then exposed to the X-ray for thirty minutes. Before the treatment, the leukocytes numbered about 106,000; six hours after, they numbered 112,000; twenty-four hours after 94,000; and forty hours after, 84,000.

In a drop of blood taken forty hours after the X-ray exposure, a larger number of free nuclei were to be seen than in the blood taken before the treatment was administered.

Repeated Exposures.

Four cases of chronic lymphatic leukæmia were exposed to X-ray over periods varying from four months to two years. In every case there was a steady fall in the number of leukocytes, until the count was normal or nearly so. The rate of decrease depended upon the frequency and length of duration of the exposures, but it also varied

with the individual. When the patient suffered a relapse the response to the treatment was slower, and after a time failed altogether. The mononuclear cells suffered relatively far more than the other cell types. Two cases of chronic splenomyelogenous leukæmia reacted to X-ray treatment in a similar way, with the exception that the myelocytes were destroyed more than any other cells.

This selective action of the X-ray in animals was first demonstrated by Linser and Helber.[7] Bozzolo[8] states that several hours after treatment the leukocyte count in splenomyelogenous leukæmia rises rapidly and then sinks again. Other observers, including Hoffman,[9] have failed to find such an increase. After long continued treatment, the X-ray may lose its effect upon the glands, spleen and blood. This indicates that the individual may acquire immunity to the X-ray.

VII. THE INJECTION OF A STRONG LEUKOLYTIC SERUM FROM A LEUKÆMIC PATIENT EXPOSED TO X-RAY INTO AN UNTREATED CASE OF LEUKÆMIA.

The serum in this case was obtained from a patient suffering from lymphatic leukæmia (Fifield), who had been under X-ray treatment at regular intervals for nearly two years. The serum, which had been tested upon leukocytes in the hanging drop and by means of animal injections, was found to be leukolytic to a high degree. The subject for the injection was a case of lymphatic leukæmia, which, for three months, had not been treated in any way and had been getting steadily worse, as was indicated by the increasing number of leukocytes, and the enlarging glands and spleen. The blood was obtained under aseptic precautions, and after being defibrinated and centrifuged the serum was drawn off and injected into the abdominal wall of the patient.

The accompanying chart illustrates the rapid fall in the number of leukocytes after each injection of the serum. The decrease was greatest at the expiration of the twenty-four to forty-eight hours following the injection. After the first injection of 4 c.c. of the

[7] *Loc. cit.*
[8] Bozzolo, abstract from *Reforma Medica*, 1905.
[9] Hoffman, *Cong. f. innere Med.*, 1905, xxii, 125.

serum the leukocytes decreased in forty-eight hours by 64,000. After the second injection of 5 c.c. of the serum, in forty-eight hours the leukocytes decreased by 34,000. After the third injection of 10 c.c. of the serum, in twenty-four hours the leukocytes decreased by 82,000. It will be observed that the leukocytes attained the original count or a higher one in periods of thirteen, nine, and six days, respectively, after the injection. Although the amount of serum injected was increased, the effect was more transitory each time. Here again is indicated a partial immunity to the leukolytic serum similar to the immunity to the X-ray itself, which may be acquired by a patient. The destruction of the leukocytes was selective, the mono-nuclear cells being the ones chiefly attacked.

In this connection may be cited the interesting experiments of Flexner[10] and of Bunting[11] upon the influence of myelotoxic and lymphotoxic sera upon the blood and blood-forming organs. These sera cause first a leukopenia, then a leukocytosis, and, finally, a return to the normal. The lymphotoxic serum caused, at first, a lymphopenia and, afterwards, a lymphocytosis, and its injection was followed by hypertrophy of the lymphatic glands.

VIII. THE NATURE OF LEUKOLYSIN AND THE MANNER OF ITS PRODUCTION.

Leukolysin is probably formed by the destruction of the leukocytes by the X-ray. This action is seen in the experiments with the leukæmic blood in test tubes exposed to X-ray, where both the fragmentation of the leukocytes and the formation of the leukolytic serum can be demonstrated. In the living subject the leukocytes in the circulating blood and the spleen may alone be the source of leukolysin; or this function may be shared by the lymphoid cells of the glands and spleen. The latter seems to us the more probable, because the formation of leukolysin and evidences of leukocyte destruction are much more marked after a given exposure of the spleen and glands to X-ray than after an exposure of the blood in the test tube. Moreover, we know that lymphoid cells, like the leukocytes, are susceptible to X-ray.

[10] Flexner, *Univ. of Penna. Med. Bulletin*, 1902, xv, 287.
[11] Bunting, *Ibid.*, 1903, xvi, 200.

The nature of this leukolysin remains in doubt. It may be an amboceptor-complement complex or a true toxine.

General Conclusions.—1. The X-ray produces in leukæmia a disintegration of the leukocytes, affecting especially the young forms, viz., the myelocytes and the non-granular mononuclear cells. A similar action, but one of less degree, takes place in leukæmic blood exposed to X-ray in vitro.

2. The serum of a leukæmic patient who has improved under the X-ray treatment, when injected into animals, causes leukopenia; when added in the hanging drop to the leukocytes of another individual it disintegrates the cells. This leukolytic action is selective, destroying first the mononuclear cells. The strength of the leukolytic action seems to be proportional to the degree of clinical improvement of the patient under the X-ray treatment.

3. The serum of a case of leukæmia which has been exposed to X-ray has a marked agglutinating action on normal red corpuscles and on other corpuscles. The degree of agglutination varies roughly with the degree of leukolysis present.

4. X-ray treatment of normal or leukæmic blood in vitro does not materially alter the phagocytic power of the leukocytes.

5. The injection of a strong leukolytic serum from a patient suffering from lymphatic leukæmia under X-ray treatment into another individual with lymphatic leukæmia, not under the treatment, caused a decided and rapid fall in the number of leukocytes. The mononuclear cells were principally affected. With repeated injections a partial immunity to the serum was established.

We take this opportunity of acknowledging our indebtedness to Drs. Billings, Bevan and Herrick for clinical material, and to thank especially Drs. Hektoen and Ricketts for their many helpful suggestions.[12]

[12] Read at the meeting of the Association of American Physicians, Washington, D. C., May 15–16, 1906.

OBSERVATIONS ON THE CYTOLOGY OF MULTIPLE NON-INFLAMMATORY NECROSIS OF THE LIVER AND ON CERTAIN RELATED DEGENERATIVE CHANGES IN CELLS.[1]

By DOUGLAS SYMMERS.

(*From the Strecker Memorial Laboratory of the New York City Hospital.*)

There has been described recently from this laboratory[2] an independent affection of the liver characterized anatomically by "multiple, irregular, circumscribed solution of liver cells without parenchymatous degeneration or coagulation necrosis, and associated with a corresponding lobular blood and bile stasis in the affected areas. It appeared that the protoplasm and later the nucleus of the cells simply wasted and dissolved, leaving a more or less well preserved reticulum with stagnant blood and bile." The condition has been observed here six times (two unpublished cases) and seems to be characterized clinically by jaundice and mental symptoms, usually delirium, followed by a rapidly fatal issue. To the lesion as a whole Oertel has applied the term "multiple non-inflammatory necrosis of the liver with jaundice." The cellular changes he designates "cytolysis."

While several observers, notably Flexner in this country and Fr. Muller in Germany, correlating chemical with morphological findings, have sought to explain certain pathological processes on the basis of the property of certain cells to elaborate digestive ferments, practically no work has been done on the structural changes brought about in the tissues by the action of such substances. Hauser[3] in 1886 kept small pieces of liver under aseptic conditions for varying lengths of time. The specimens gradually softened.

[1] This study was aided by an appropriation from the Rockefeller Institute for Medical Research.

[2] *Jour. Med. Research*, 1904, xii, 75. *Jour. Exper. Med.*, 1906, viii, 103. *Collected Proc. N. Y. Path. Soc.*, new series, 1905–6, v, 143.

[3] *Arch. für exper. Path. v. Pharm.*, 1886, xx, 162.

Microscopically the cell form was found to be preserved to a late date, the nuclei were degenerate and the cytoplasm appeared granular. Waldvogel[4] recently repeated these experiments with like results. Both these observers, however, were working on problems which did not intimately concern the morphology of autolysis and consequently their description of the cellular changes is lacking in detail.

The present communication has to do, first, with a detailed description of the cell changes in multiple non-inflammatory necrosis of the liver and with related cell changes in other conditions, and, second, with the description of a variety of cell degeneration likewise affecting the liver and, I believe, generically allied to that of Oertel's lesion but presenting distinctly different characteristics, both general and cytological.

In multiple non-inflammatory necrosis the individual liver cells are large and, although irregular in outline, the various forms are well preserved. The nuclei are relatively large and occupy, as a rule, a central position in the cell body, being pushed aside only in those instances in which the cytoplasm has been the seat of fat deposit. The cytoplasm appears pale and washed out and stains poorly. The nucleus, relatively much richer in substance, stains correspondingly deeply; its shape for the most part is well preserved and the nuclear membrane appears to be intact and stains deeply, while immediately within the membrane are numerous large and small, deeply staining granules which follow the circumference of the nucleus. The center of the nucleus is pale and turbid and not infrequently is traversed by fine chromatin bands which appear to radiate from the relatively large, rounded, deeply staining nucleolus or nucleoli. In those cells in which the nucleus appears only slightly disintegrated the cytoplasm is correspondingly well preserved.

The change in the cytoplasm appears primarily to be a simple solution of the hyaloplasm with retention of the spongioplastic network, giving the cell a finely honeycombed appearance. In places the meshes of the spongioplasm are partly occupied by minute bile particles; in other places certain alveoli seem to have coalesced to

[4] *Virchow's Arch.*, 1904, clxxvii, 1; *Deut. Arch. für klin. Med.*, 1905, lxxxii, 437.

form a large vacuole for the reception of aggregations of such particles. In other places the cytoplasm shows large vacuoles which, in fresh sections stained in Sudan III, reveal the presence of neutral fats.

A further step in the destruction of the cell after the hyaloplasm has been dissolved out is represented by shrinkage of the spongioplasm, followed by enlargement and distortion of the cell body and partial or complete nuclear destruction. The fine spongioplastic network may be retained in places and lost in others. In the end all that remains is a wrinkled but otherwise intact cell membrane enclosing, in some instances, a few coarse, irregularly distributed reticular fragments and occasional fat droplets or aggregations of bile.

It is found, however, that cell changes simulating those just described are not confined to multiple non-inflammatory necrosis of the liver. In the exudate in resolving pneumonia, in the cells that lie free in the alveoli in chronic cyanosis of the lungs, and in cerebral softening, similar structural changes are apparent, brought about, it is believed, by solvent action.

The solution of the red cells can be traced both in the capillaries and smaller vessels and in the alveoli, especially in chronic cyanosis of the lungs. The first change appears to consist of partial solution of the hæmoglobin manifested by a diminution of the avidity with which the cell takes up the acid stain. The corpuscle stains a dull pink or dirty yellow and becomes opaquely granular. From this point the staining qualities can be traced through certain gradations in color until finally the cell stains very faintly or not at all, appearing as a definitely rounded body with an intact cell membrane which encloses a pinkish or bluish or grayish opaquely granular material, the cell varying in diameter from five to twelve microns or even more. Finally the altered cell substance is completely dissolved out and all that remains is the cell membrane. This structure frequently retains its rounded shape, but occasionally appears wrinkled —the "shadow" or "ghost" cell. In the pulmonary alveolus the pale, definitely rounded, non-nucleated cells constitute a characteristic feature, the peculiar constitution of the cytoplasm being very striking. It is apparent, also, that after being extruded into the

alveolus the corpuscle often increases in size, an effect due, no doubt, to a variety of factors.

In the white cells certain individual morphological variations are presented. The leucocytes earliest affected are comparable to the large lymphocytes; the polymorphonuclear cells, while not infrequently involved, appear to be relatively immune or at least more resistant. The first visible change in the leucocytes consists of swelling of the cytoplasm, which becomes cloudy and stains a dull red with eosin. It then appears to lose entirely or almost entirely its affinity for the acid dye, staining faintly blue or pink. The cells become more or less irregular both in shape and size, although the continuity of the enclosing membrane is practically always preserved. At this stage the cytoplasm shows a distinct reticular arrangement with the formation of empty alveoli. The nuclei display various degrees of disintegration and are much distorted. The further destruction of the cell may be accomplished, however, in different ways and the various steps cannot well be followed. Sometimes the cytoplasm at the periphery is lost, a few irregular clumps being arranged around the rapidly disintegrating nucleus. Or the nucleus may be found lying next the cell membrane, where frequently it becomes flattened or cresentic in outline, while the cell body is made up of irregularly scattered reticular remnants. Finally the nucleus is lost and the cell appears either as an irregularly rounded body enclosing a few reticular fragments or as a mere shell.

In chronic encephalitis with extensive softening the leucocytes in the softened areas and, to a less extent, those in the better preserved parts of the brain, show all varieties of the degenerative changes just described that lead to the exposure of the cell reticulum. In addition there are to be seen numbers of large, very pale leucocytes whose outline is irregular but whose cell membrane is preserved. The cytoplasm appears to be made up of opaque, structureless material and the nucleus is markedly degenerate. In some of these cells the nucleus has disappeared and the cell membrane encloses opaque, amorphous material only, which may be scattered evenly over the surface or arranged irregularly in clumps. In still other cells the membrane encloses nuclear fragments and small

collections of debris. In the end the cell membrane alone remains.

This peculiar appearance of the cell may be likened to that of the cytolyzed red cells already described, or more aptly still to certain structural changes in the liver cells to be described later.

Related changes can be followed in the alveolar epithelium in pneumonia and in chronic cyanosis of the lungs. These cells, as they lie attached to or partially detached from the alveolar wall, appear as large, palely staining, flattened or elongated bodies with pale, rounded or oval nuclei which enclose rather deeply staining nucleoli. The cytoplasm presents a distinct reticulum and the hyaloplasm is not visible. After the cell is completely detached and lies free in the alveolus it often becomes swollen and irregular in shape, but retains otherwise the general characteristics just described. Its identification in this position can usually be determined by the fact that its nucleus encloses a nucleolus, by the shape of the cell and by the occurrence of large, golden brown pigment particles, although similar pigment masses now and then can be seen lying upon or within cytolyzing leucocytes.

In connection with the general question of cytolysis I wish to draw attention to a form of cell degeneration occurring in the liver, for which, assuming that the morphological interpretation is correct, because of the character of the cellular changes presented, I would suggest the term "cytolysis granulosa." Since the condition was first detected I have looked for it in about two hundred cases and have been able to find it present seven times as a widely spread, diffuse affection and several times as a more restricted process. Neither the clinical histories of the respective patients nor the microscopical findings in the different organs throw much light on the changes in the liver cells. Three patients presented well-marked anatomical evidences of syphilis. The gross appearance of the liver was not regarded in any case as at all remarkable.

In three instances the autopsy was performed within four or five hours after death. Specimens were preserved in 5 per cent. formalin solution and examined in frozen or celloidin and paraffin sections. The change appears to show to best advantage when sections are deeply stained in Böhmer's hæmotoxylin and examined under the oil immersion lens.

In this condition the liver cells appear for the most part to be arranged in definite rows, closely packed together; the cell outline is unusually distinct and the normal shape almost always is retained. The intercolumnar vascular and lymphatic channels are wide. Under the oil immersion lens the cells everywhere appear pale and of average size. The cell membrane stains deeply and stands out prominently against the pale cytoplasm. The nucleus almost uniformly is enlarged out of proportion to the size of the cell as a whole and is usually rounded, occasionally indented, in most cells lying in the center, in some being pushed to one side. In what seems to be the initial step in the process the cytoplasm looks pale and made up of turbid, granular looking, structureless material evenly but thinly distributed over the surface of the cell, and composed apparently of a combination of altered hyaloplasm and broken-down reticulum. The nucleus now shows beginning central turbidity with small chromatin granules arranged peripherally. A nucleolus—sometimes more than one—may be present. In cells still more badly damaged this hazy, dirty looking material is arranged in irregularly scattered clumps, while between the clumps are finer, hazy, granular looking particles. At this stage the nucleus is very pale and the center very turbid, but the nuclear membrane still stains deeply and is intact. A few small chromatin granules are visible within the body of the nucleus. Toward the end the nucleus shrinks in size and retreats toward the periphery of the cell where it seems to be supported partly by the cell membrane and partly by clumps of debris; its center now is extremely turbid but the enclosing membrane still is very definitely outlined. Finally, the cell contents disappear completely and the cell membrane alone remains.

In most cases moderate bile imbibition is to be observed. It occurs in two forms: First, as very finely divided particles scattered over certain cells and, second, as aggregations of such particles lying in large vacuoles in the cytoplasm. The vascular and lymphatic channels lying between the rows of liver cells are usually greatly dilated, their endothelial lining being easily distinguished. The vascular channels contain a few pale red cells, shadow corpuscles, small numbers of leucocytes and debris. Sections stained in Sudan III reveal, in most cases, fat in small amount, appearing

mostly as rather large rounded masses, but occasionally as very minute particles lying in the cytoplasm.

The peculair material replacing the cytoplasm of the liver cells is insoluble in chloroform, ether, alcohol, equal parts of ether and alcohol, 1 per cent. sodium hydrate solution and 2 per cent. acetic acid solution. The last substance, however, renders it cloudy, so that the cell membrane is obscured. It is unaffected by prolonged exposure to 1 per cent. osmic acid solution, except to a very slight degree, a few small, blackish spots appearing here and there.

From these observations the following conclusions may be drawn:

1. Those pathological processes that appear to depend upon cytolysis present at least two definite types of structural changes in cells—the one characterized by rapid, simple solution of the hyaloplasm with preservation of the cell reticulum as, for example, in multiple non-inflammatory necrosis of the liver; the other by alteration of the hyaloplasm and reticulum with the formation of amorphous material, both the cell and nuclear membranes remaining very resistant, as in the liver cells just described. Extreme tenacity of the cell membrane appears to be the most constant single feature of all forms of cytolysis thus far recognized.

2. Polychromatophilia is associated with solution of the hæmoglobin of the red cells, especially in those conditions which are attended by destruction of blood. It appears not improbable that this peculiar reaction of the cell may represent a physiological as well as a pathological condition. As a physiological process it is conceivable that the polychromatophilia apparent in the earlier developmental stage of the red cells represents an attempt on the part of the organism to dispose of an excess of such cells by solution or that the cells have not yet received their full supply of hæmoglobin.

In closing I wish to express my appreciation of the interest shown in this work by Dr. Horst Oertel.

EXPERIMENTAL ATRESIA OF THE URETER.

BY TORALD SOLLMANN, W. W. WILLIAMS AND C. E. BRIGGS.

(From the Pharmacological Laboratory of Western Reserve University, Cleveland, Ohio.)

The effects of the complete and permanent occlusion of one ureter have been studied by numerous investigators. The older literature, which is summarized by Lindemann,[1] bears mainly on the morphological changes occurring in the kidney, the ureter of which was ligated. The more recent experiments[2] have dealt particularly with the changes observable in the opposite kidney, with the object of investigating the existence of auto-nephrotoxins. The functional changes produced in the kidney by ligation of its ureter have been touched upon only incidentally. This last condition suggested the present research, but in the course of this study we also controlled the morphological observations of previous investigators.

OPERATIONS AND MORPHOLOGICAL CHANGES.

Operations.—Four dogs were operated on August 7, 1903, and the right ureter ligated about 5 centimeters from the pelvis of the kidney. Dog (1) died after 31 days. Dog (2) sickened about the 72d day, and was killed on the 74th day. On the 107th day dogs (3) and (4) were re-operated under ether, and urinary fistulæ were established with the right kidneys. Dog (4) died 9 days later, or 116 days after the primary operation. Dog (3) was killed on the 185th day (the 78th day after the second operation).

The kidneys of all dogs showed more or less similar gross and microscopic changes with the exception of (4), in which the lesions were masked by a generalized renal infection.

Operated Kidneys.—The kidneys on the ligated side were enlarged and the pelvis contained from 88 to 250 c.c. of fluid. Minus

[1] Lindemann, *Zeitsch. f. klin. Med.*, 1898, xxxiv, 299.

[2] Pearce, *Univ. of Penna. Med. Bull.*, 1903, xvi, 217. Ames, *Jour. Path. and Bact.*, 1903, x, 265.

the fluid, the weight of the operated kidney was only one-fourth to two-thirds of that of the sound kidney. The capsule of the kidney as well as Bowman's capsule were slightly thickened. Most of the glomeruli were shrunken and many were replaced by fibrous tissue. The epithelial cells of the convoluted tubules showed cloudy swelling, necrosis and pressure atrophy. Most of the tubules were obliterated. The collecting tubules ran generally parallel to the cortex and showed slight dilatation in places. The interstitial tissue was increased, with slight round-cell infiltration. The arteries showed various degrees of endarteritis and periarteritis.

Sound Kidneys.—The kidneys on the non-ligated side were always slightly enlarged and microscopically showed slight cloudy swelling and moderate congestion especially of the medulla.

PROTOCOLLS.

Dog 1.—The right ureter was ligated 5 cm. from the kidney on August 7, 1903. The dog, without apparent cause, died October 7, 1903 (31 days).

Autopsy.—Putrefactive odor. Very small external scar. Internally small adhesions. Left kidney seems normal. Pelvis of the right kidney distended. Righ ureter dilated to about 1 cm. in diameter.

Sound kidney weighs	31 grams.
Operated kidney weighs	124 grams with liquid.
Operated kidney weighs	21 grams without liquid.

Therefore, 103 grams of liquid.

The right kidney consists of a sac with walls about 2 mm. in thickness.

Microscopic Examination.—Right kidney: The capsule is slightly thickened. The glomeruli show moderate thickening of Bowman's capsule. The capsular spaces are not dilated. Some tufts are considerably shrunken, with increased growth of fibrous tissue; and an occasional tuft is replaced by fibrous tissue. The convoluted tubules are for the most part obliterated, although there are occasional normal ones and others are lined with only a small fringe of ragged protoplasm containing apparently healthy nuclei. The collecting tubules are all pressed to one side and their general direction is parallel to the kidney capsule. Most of them have slightly flattened epithelial cells with nuclei in good condition. There is apparently slight dilatation of some. The blood vessels show moderate congestion. The arteries show slight internal thickening and more marked

periarteritis. The interstitial tissue is increased throughout, with a few small areas of round cell infiltration.

Left kidney shows slight cloudy swelling and moderate congestion especially of the medulla.

Dog 2.—The right ureter was ligated 5 cm. from the kidney on August 7, 1903. Reported sick on October 21. On October 23, dog receives morphin at 8:15 A. M. and ether at 8:45. Weight 10 kg. Cannula placed in left ureter at 9:24. Cannula placed in right ureter at 9:40. Urine flowed very slowly from the right kidney and ceased entirely at 10:15. At 10:30 A. M. 100 c.c. of 10 per cent. crystallized sodium sulphate was injected slowly into femoral vein. Dog died suddenly without assignable cause. Lived 74 days.

Sound kidney weighs	42 grams.
Operated kidney weighs	150 grams with liquid.
Operated kidney weighs	20 grams without liquid.

Therefore, 130 grams of liquid.

The pelvis of right kidney is markedly dilated.

Microscopic Examination.—Right kidney. The capsule is slightly thickened. The glomeruli show slight thickening of Bowman's capsule. Many capsular spaces are dilated and the tufts occupy one-third to one-half the space, the remaining portion filled with finely granular material. Some of the tufts are shrunken and many are obliterated. Nearly all of the convoluted tubules are obliterated or represented by a mass of nuclei surrounded by a little granular protoplasm. A few are considerably dilated and lined with large cuboidal epithelial cells. The collecting tubules run more or less parallel to the cortex. Many are obliterated and the majority of the remaining ones show varying degrees of dilatation. Many are filled with casts. The interstitial tissue is everywhere increased with small areas of leucocytic infiltration. There are a few small areas in the cortex that look like healed infarcts. The arteries show slight intimal and more marked adventitial thickening. Slight congestion. The pelvic lining is thickened and consists of several layers of rather large polygonal cells.

Left kidney as in dog No. 1.

Dog 3.—The right ureter was ligated 5 cm. from the kidney on August 7, 1903. On November 25 (107 days after the primary operation) a second operation was performed and 88 c.c. of urine were gently squeezed out and a urinary fistula was established. On the next day the dog was fairly lively. No food was given until November 30. On December 4 the skin was gaping, but the dog was lively. Ureter fistula was not patulous to a probe which could

be introduced only 5 cm. On February 11, 1904 (185 days after the primary operation and 78 days after the second) the dog was very well, although the abdominal wound was not quite healed. The fistula was moist. Could pass probe to kidney. 9:10 A. M. 2 c.c. 4 per cent. morphin, 9:30 ether. Right kidney found shrunken and empty. Cannulæ were placed in the ureters. The right remained perfectly dry; the left showed good secretion, as follows:

From 10:45 to 11:07 the average secretion of urine was 0.2 c.c. per minute.

From 11:07 to 11:10 225 c.c. of 5 per cent. cryst. sodium sulphate at 40° C. was injected into the femoral vein.

From 11:07 to 11:15 average secretion of urine was 1.75 c.c. per minute.

From 11:15 to 11:25 average secretion of urine was 3.6 c.c. per minute.

The right cannula remaining dry, it was reinserted nearer the kidney. It filled with a little bloody serum which could not have been more than 2 or 3 c.c. and no fluid was secreted during the experiment.

From 11:25 to 11:37 average secretion of urine was 2.6 per minute.

From 11:37 to 11:42 average secretion of urine from the left kidney almost ceased, being 0.2 c.c. per minute (probably due to shock).

From 11:42 to 11:44 second injection of 200 c.c. sodium sulphate solution. Left kidney starts secreting at once.

From 11:45 to 11:50 average secretion of urine was 2.5 per minute.

From 11:50 to 11:55 average secretion of urine was 2.5 per minute.

From 11:55 to 12:06 average secretion of urine was 2.5 per minute.

The left ureter was clamped at 12:08. Between 12:10 and 12:12 a third injection of 200 c.c. sodium sulphate solution was made. At 2 P. M. only 2 drops had escaped from the right ureter. The dog was killed.

Sound kidney weighs 43.2 gm.

Operated kidney weighs 9.6 gm.; about 4.5 mm. thick.

The right kidney is small, measuring 30 mm. in length, 16 mm. in breadth, and 8 mm. in thickness. On section it is very dense and the pelvis contains no fluid. The left kidney measures 41 mm. in length, 31 mm. in breadth, and 21 mm. in thickness.

Microscopic Examination.—Right kidney. The capsule is somewhat thickened. The glomeruli show slight thickening of Bowman's capsule. Many capsular spaces are dilated and the tufts are shrunken. A few glomeruli are obliterated and replaced by fibrous tissue. The convoluted tubules are mostly obliterated, some are represented by a confused mass of epithelial cells with their nuclei showing varying degrees of degeneration. In small discrete areas, usually near the cortex, are a few tubules lined with large epithelial cells and apparently in good condition. The collecting tubules are mostly obliterated and some of the remaining ones are dilated and contain casts. The interstitial tissue is increased throughout with a few small hæmorrhagic and scattered areas of leucocytic infiltration in it. The blood vessels are moderately congested and show marked adventitial thickening. The pelvic epithelium is thickened and consists of several layers of large cells.

Left kidney. The glomeruli are larger than normal; the tubules are apparently larger, with normal epithelial cells. Moderate congestion.

Dog 4.—The right ureter was ligated 5 cm. from kidney on August 7, 1903. On November 25 (107 days after the primary operation) a second operation was performed and 88 c.c. of urine were gently squeezed out and a urinary fistula established. On the next day the dog was fairly lively. No food was given until November 30. The dog gradually became sick and at noon of December 4 was comatose and died in the afternoon (116 days after the primary operation and 9 days after the second). The dog was kept frozen and was autopsied on December 7, 1903. No peritonitis. Right kidney was enormously enlarged with very thin walls and the pelvis was distended with 225 c.c. of reddish, turbid fluid, containing some pus. No sufficient cause of death could be assigned.

Microscopic Examination.—Right kidney. The capsule is much thickened and numerous vessels connect it with the cortex. Bowman's capsule is also considerably thickened. Most of the glomeruli are replaced by fibrous tissue, although a few tufts remain which are much shrunken and encroached upon by fibrous tissue. Practicallv no tubules are to be seen. The interstitial tissue is increased. The arteries show varying degrees of endarteritis and several rather large ones are thrombosed. There are a few small hæmorrhages. The greatest part of the sections is composed of necrotic tissue with here and there indefinite remains of glomeruli and tubules and everywhere marked infiltration with polymorphonuclear leucocytes predominating although no definite abscesses are to be seen.

CHEMICAL EXAMINATION OF THE FLUIDS IN THE OCCLUDED URETERS.

The results of these examinations are presented in Table I, the analytical methods appearing in the appended notes. Column III gives a summary of the fluid obtained after the first operation in the four dogs. The fluid from the left ureter of the congenital atresia reported by Dr. Allen[3] is also included in this table.

1. *Duration of the Atresia in the Human Case* (see page 82 of the JOURNAL). —In the thirteen years which elapsed since the establishment of the right kidney fistula, no urine was voided from the bladder, so that the left ureter must have been completely occluded for at least this period of time. It is probable, however, that the atresia was congenital, *i. e.*, that it persisted for 16 years.

2. *Quantity of Fluid.*—The quantities are only approximate, generally somewhat too low.

3. *Color of Human Fluid.*—After the precipitation of the proteid, the fluid has a light straw, serum color, which is slightly darkened by nitric acid and by heating with soda.

4. *Color of Dogs' Urine, 3 and 4.*—This is not altered by precipitating the proteids.

5. *Color of Second Accumulation of Fluid in Dog 4.*—This is fairly deep red, with an abundant slimy grayish red precipitate. The filtrate, after the removal of the proteids, is very faintly colored.

6. *Odor of Human Fluid.*—This is faint, somewhat like serum, on heating. It is not urinous, even on boiling with soda.

7. *Specific Gravity.*—This was taken with a picnometer in the human case, with areometers in the dogs.

8. *Depression of Freezing Point.*—Determined by Beckmann's apparatus, $\frac{\Delta}{\text{NaCl per cent.}} = 0.86$ in the human case.

9. *Total Solids.*—10 c.c. dried at 110° C.

10. *Ash.*—Incineration of 10 c.c. This is apt to show a trifle higher than it should, if the incineration is incomplete.

11. *NaCl.*—Incineration of 5 to 10 c.c. with $NaNO_3$ and Na_2CO_3, solution in HNO_3 to neutralization; titration with $AgNO_3$. Chromate indicator.

12. *Non-chloride Ash.*—Difference between total ash and NaCl. This is apt to be too high, see (10).

13. *SO_4.*—Gravimetrically with barium, in 20 c.c.

14. *P_2O_5.*—The determination of SO_4 and P_2O_5 is not very exact on account of the small quantity.

15. *Organic Solids.*—Difference between total solids and ash.

16. *Total Coagulable Proteid.*—10 to 50 c.c. of the fluid are acidulated with acetic acid and boiled with an equal volume of saturated sodium sulphate or chlorid. The precipitate is washed with water, alcohol and ether, dried at 110° C. and weighed.

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[3] See page 82, this number of the JOURNAL.

TABLE I.

ANALYTICAL DATA OF THE FLUIDS OBTAINED FROM THE LIGATED KIDNEY.

Column I.	II.	III.	IV.	V.	VI.	VII.	VIII.
Experiment.	Human.	Summary of Dogs, 1 to 4. First Operation.	Dog 1.	Dog 2.	Dog 3.	Dog 4. First Operation.	Dog 4. Second Operation.
Time between occlusion of ureter and collection of fluid.	At least 13 years (1).	31 to 107 days.	31 days.	74 days.	107 days	107 days.	78 days.
Quantity of fluid (2).	200 c.c.	88 to 130 c.c.	103 c.c.	130 c.c.	88 c.c.	88 c.c.	225 c.c.
Color.	Faint amber, not urinous (3). Turbid.	Yellow urinous.	Fairly deep yellow.	Light yellow.	Quite deep yellow (4).	Deeper than with Dog 3.	Bloody, not urinous (5) Turbid.
Odor.	Faint, serum (6).	None.	Putrefactive.	None.	None.	None.	None.
Reaction to litmus.	Neutral.		All the dogs' urines have a slightly alkaline reaction.				
Sp. gr. (7).	1.0103	1.008 to 1.009	1.008	1.009	1.009	1.0085	1.018
Depression of freezing point	0.715°C (8)	—	—	—	—	—	—
Total solids % (9).	—	1.8 to 2.1	2.115	—	2.035	1.800	5.805
Ash % (10).	0.895	0.8 to 0.95	0.956	—	0.805	0.845	—
Cl, as NaCl % (11).	0.83	0.68 to 0.75	0.75	0.73	0.72	0.68	0.52
Non-chloride ash % (12).	0.065	0.085 to 0.206	0.206	—	0.085	0.165	—
SO_4 % (13)	—	0.021 to 0.036	—	—	0.021	0.036	—
P_2O_5 % (14).	0.046	0.033 to 0.065	—	—	0.033	0.065	—
Organic solids % (15).	—	0.955 to 1.230	1.159	—	1.230	0.955	—
Total Coagulable Proteid % (16)	0.25	0.390 to 0.858 Mean=0.74	0.858	0.657	0.820	0.390	3.932
Globulin (17).	Present.	Present.	—	—	Present.	0.175%	Present.
Albumin (18).	Present.	Present.	—	—	Present.	0.215% (19)	Present.
Non-coagulable Proteids (20).	Absent.		Probably absent in all the dogs' urines.				
Non-proteid Nitrogen % (21).	—	0.056 to 0.126 mean= 0.103	0.080	0.126	0.056	0.126	—
Urea % (22).	0.814 (24)	0? to 0.165 mean= 0.112	0.068	0.165 (25)	0?	0.156	—
Ammonia % (23).	Present.	0.0025 to 0.0054	0.0025	0.0054	—	—	—
Non-urea nitrogen % (26)	—	0.047 to 0.056	0.047	0.048	0.056	0.053	—
Reducing substance.	Small quantity.	—	—	—	—	—	—
Sediment.	Cellular, abundant.	—	Round cells of various types, largely degenerated.	A few blood and other cells (leucocytes?) largely degenerated.	—	—	Very abundant, many blood cells and considerable fibrin

17. *Globulin.*—Addition of equal volume of saturated ammonium sulphate.

18. *Albumin.*—Boiling the acidulated filtrate of (17). The Albumin : Globulin :: 1.23 : 1, in Dog 4.

19. The percentage of albumin is obtained by subtracting the globulin from the total proteid.

20. *Non-coagulable Proteid.*—Biuret test of filtrate of (16).

21. *Non-Proteid Nitrogen.*—Kjeldahl on filtrate of (16).

22. *Urea Nitrogen.*—Folin's method on filtrate of (16).

23. *Ammonia Nitrogen.*—Folin's method on filtrate of (16).

24. *Urea in Human Fluid.*—This was not determined by Folin's method, but by estimating the nitrogen in the boiled filtrate. This gave a small precipitate with phosphotungstic acid; some proteid had therefore probably escaped precipitation, and the figure for urea is too high, and unreliable. The presence of urea was shown qualitatively by the effervescence of a hypobromite solution.

25. *Qualitative Tests for Urea were Positive.*

26. *Non-Urea Nitrogen.*—Difference between (21) and urea nitrogen.

SUMMARY, DISCUSSIONS OF THE RESULTS, AND CONCLUSIONS.

Previous investigators have found that complete occlusion of the ureter may lead either to hydronephrosis or to atrophy. In Lindemann's series of six dogs, for instance:

Two animals showed simple hydronephrosis, three animals showed simple atrophy, and in one animal the kidney was slightly enlarged and the ureter and pelvis dilated, but fluid was absent. In his series of four rabbits, all showed hydronephrosis.

The result, whether hydronephrosis or atrophy, is evidently not determined by the time elapsing after the operation. Lindemann found that the intrapelvic pressure resulting from the ligation obliterates the lumen of the vessels, first of the veins and subsequently of the arteries; but that this is compensated by an increase of the collateral blood supply through the capsule, the degree of this compensation determining the presence or absence of hydronephrosis. If the blood supply is free, the fluid after tapping will accumulate again and again.

It is somewhat remarkable that all of our dogs showed hydronephrosis after the first operation. The results of establishing a urinary fistula differed in the two cases in which it was tried: The fluid did not re-form in Dog 3 even when sodium sulphate was

injected; whereas in Dog 4, a very abundant quantity of fluid reaccumulated spontaneously; but it differed notably in composition from the original fluid, having more the character of a purulent inflammatory exudate.

The histological changes consist in necrosis of the renal cells, obliteration of the glomeruli, increase of connective tissue, and endarteritis and periarteritis. Different areas in the same kidney are affected in very different degree, some areas appearing almost normal. The glomeruli are generally less altered than the tubules. The collecting tubules are generally displaced so as to run parallel to the surface; many are dilated. The changes correspond closely to those described by Lindemann.

The sound kidneys showed slight hyperæmia and hypertrophy, but no necrosis. This corresponds with the findings of Pearce and of Ames.

The uniformity in chemical composition of the fluid obtained, after the first operation, from the four dogs, as shown by Column III of Table I, is very striking, and points to a uniform origin by a process which is but little affected by the interval elapsing after the operation. The specific gravity, total solids and proteids correspond to those of a very dilute lymph, being but a trifle above those of cerebro-spinal fluid and aqueous humor, and much lower than those of serum, lymph and most cystic fluids (the proteid content of the latter being generally from 2 to 6.5 per cent.). The human fluid (Column II) which had remained in the kidney for a very long time had a particularly low proteid percentage; while that of the second fluid of Dog 4 (Column VIII) was very much higher; this last fluid having a pronounced inflammatory character and being of recent formation.

The absence of notable amounts of the specific urinary constituents is particularly important.[4] Odorous principles are entirely

[4] The research was originally undertaken at the suggestion of one of us (Briggs), to decide whether it would be possible to re-establish the function of the kidney after temporary ligation of the ureter. The answer must be in the negative, at least when a considerable interval elapses before the establishment of the fistula.

absent. Urinary pigments appear to be present in the four dogs' urines, but absent from the human case,[5] and after the second operation in case of the dogs. It seems fair to assume that the pigments were secreted shortly after the ligation, when the kidneys were still functional, and that they were reabsorbed with extreme slowness.

Urea was present in all the fluids, but its quantity was very small in the dogs, and probably in the human case. It is on the whole somewhat greater than in the serum (0.103 per cent., in place of 0.05 per cent.), but the difference may be within the analytical error. The same applies to the ammonia, phosphates and sulphates.

An important difference between these fluids on the one hand and blood serum, lymph and ordinary exudate on the other, lies in the higher contents of chlorid, and the consequently greater molecular concentration. The ordinary chlorid content of body fluid varies between 0.55 and 0.70 per cent., mean about 0.6 per cent (as NaCl), while that of the first kidney fluid, in the dogs, varied between 0.68 and 0.75 per cent., mean 0.725 per cent.; that of the human fluid was 0.83 per cent., that of the second fluid of Dog 4 only 0.52 per cent. The depression of the freezing point in the human case was 0.715° C., as against the normal value, for human serum, of 0.491 to 0.562. (Possibly the blood of this patient had a higher concentration than normal, since uræmia existed.)

The high chlorid percentage has evidently no relation to the length of time during which the fluid sojourned in the kidney. It is probably to be explained by the relatively slow absorption of this ion from the kidney pelvis. It is also to be remarked that cerebrospinal fluids generally have a somewhat high chlorid content (0.573 and 0.6 per cent.),[6] but this never reaches the height of these ureteral fluids.

Conclusion.—The fluid accumulating in the kidney, after unilateral ligation of the ureter, consists in the main of a transudate,

[5] Lindemann (*l. c.*) and Starling (Schaefer's Text-book of Physiology, 1898, i, 650) state that the fluid in atresia of the ureter is of light color, low specific gravity, and very low urea content.

[6] T. Sollmann, *Journ. Am. Med. Assoc.*, June 6, 1903.

poor in proteids, but somewhat enriched in chlorids and perhaps in urea, phosphates and sulphates. A small quantity of pigment is also retained. The fluid is probably formed by filtration through a filtering surface which is not freely permeable to proteids. A process of reabsorption also goes on simultaneously, in such a manner that the soluble solids are somewhat increased (perhaps to counterbalance the osmotic value of the serum proteids). There is no evidence that the remaining specific renal elements play any part in the formation of this fluid, although this part is not excluded.

We are indebted to Dr. Wm. T. Howard, Jr., for the preparation of the histological material and for valuable criticism.

HYDRONEPHROSIS OF RIGHT KIDNEY, CONGENITAL ATRESIA OF LEFT URETER, AND MARKED ATROPHY OF LEFT KIDNEY.

By DUDLEY P. ALLEN, M.D., AND HENRY P. PARKER, M.D.,
CLEVELAND.

The following case is sufficiently unusual to warrant its being recorded, and hence we desire to report it briefly.

In May, 1889, one of us (Allen) was called to see the patient, C. D., æt. 2½ years. The abdomen was markedly enlarged, the enlargement evidently being due to a collection of fluid filling the whole right side. An aspirating needle had been inserted on two different occasions, and the physician was positive that he had obtained the hooklets of an echinococcus. A diagnosis was therefore made of an echinococcus cyst of the liver and an operation was undertaken.

On May 4 the patient was anæsthetized and an incision parallel to the ribs was made upon the right side. A cyst was encountered from which a clear fluid was evacuated. This fluid resembled what is sometimes seen in a cyst of the echinococcus. It was preserved but no hooklets could be found in it. The amount of clear fluid removed was small, and it became at once apparent that there was another collection of fluid immediately below it. On carrying the incision more deeply, a cyst was opened containing a large amount of fluid, amber in color and having a urinary odor. After evacuation of the fluid, on insertion of the finger, the calices of the kidney could be distinctly palpated. It became evident, therefore, that the cyst was a greatly distended kidney. Finding this to be true an effort was made to extirpate it by shelling it out of the surrounding tissues. After it had been partially loosened, it was found, however, that the child's strength would not endure the completion of the operation, so that the portion of the cyst wall which had been set free, and which was made up of kidney tissue, was quickly removed, hæmorrhage being controlled by the application of an over-and-over

suture as the tissue was cut away. The cavity was then packed with gauze, a drainage tube being inserted outside of the gauze. The patient recovered from the operation and for a few weeks occasionally passed a few drachms of urine *per urethram*. After this, all the passage of urine by the natural channel ceased, and up to the time of the patient's death, February 3, 1902, he passed no urine whatever except through the opening in the side. The incision in the cyst wall gradually contracted, so that later a silver tube was fitted into it and to this was attached a rubber bag. The boy's growth was normal, he felt well and attended school. Aside from the leakage of urine around the tube in the side, which gave him a good deal of annoyance, and the fact that he could wear nothing to catch the urine at night, he seemed in normal health. This condition continued until June, 1900. At that time the tube began to give him considerable discomfort, the urine no longer flowed freely through it, but escaped around it. Consequently, on July 13, 1900, the patient was anæsthetized, the opening was somewhat enlarged and repacked. The patient was not seen again until the middle of January, 1902. At that time he came to the office looking extremely pale, and his mother said that he had suffered from severe hæmorrhage from the nose and there had also been a considerable discharge of blood from the side. In addition to the extreme pallor there was slight œdema of the face and legs. The heart action was greatly increased, and on examination it was found that the heart was much enlarged, the apex being displaced outward and downward. There was also a blowing, systolic murmur heard at the apex. The hæmorrhage from the kidney continued, and on February 3, 1902, the boy died, having developed marked symptoms of uræmia. An autopsy was made on the morning of February 4, conducted by one of us (Parker). The following is the report upon the case:

Anatomic Diagnosis.—Complete stenosis of right ureter at its renal end; hæmorrhagic pyelo-nephrosis with marked distention of right kidney; fistula communicating between the lower end of the right renal pelvis through the abdominal wall; hypertrophy of right kidney with acute and chronic interstitial nephritis and pressure atrophy; congenital atresia of left ureter; hydro-ureter; chronic

interstitial nephritis with marked atrophy of left kidney; slight dilatation of the right ureter associated with valve-like folds of the mucosa; hypertrophy of heart; congestion of lungs and spleen.

The protocol will be limited to the genito-urinary apparatus:

The external genitals are normal. The bladder is small, being 5 cm. long and 3 cm. in its greatest diameter. The right ureteral orifice is normal in position, and is readily found with the probe which passes into the ureter. The right ureter has two fusiform dilatations, the first is just below the pelvis and is 5 cm. long, the second is in the middle third and is 3 cm. long. The ureter is 18 cm. long, its greatest diameter is 12 mm., and its least diameter, 4 mm. The lumen is irregular, but is patulous from the bladder to within 1 cm. of the pelvis at the lower pole of the kidney, where it is obliterated by a fan shaped mass of fibrous tissue. On section, valve-like folds are found just below the fusiform dilatations and the mucosa is somewhat thickened. The ureter is in general empty, but here and there is a small amount of granular material on the mucosa. The right pelvis is markedly distended, and is situated behind the kidney extending 3 cm. above its upper pole. It measures 12 cm. by 6 cm. by 4 cm., and is filled with fluid. At its lowest point the pelvis communicates with a fistulous tract which opens exteriorly in the right flank. On section, the pelvis is covered with a thin, grayish-yellow pseudo-membranous exudate, and the fluid is found to be hæmorrhagic with a urinous odor. The right kidney is embedded in fat and the lower pole is closely adherent to the abdominal parietes at the side of the fistula, causing it to be lifted forward and to the right. The exact measurement of the kidney is impossible, but it appears to be larger than normal and rests upon its own dilated pelvis. The other relations of the kidney are normal. The capsule is markedly adherent and the surface of the kidney is finely granular. Its consistence is somewhat increased. On section, it is pale and somewhat opaque and here and there is marked with small yellowish points. There are no large abscesses to be found. The kidney tissue is distorted, and varies considerably in thickness, from 0.25 cm. to 2 cm. The thickest portion is the lower half of the anterior aspect, while on the posterior surface in places there is only a thin shell of tissue remaining. In most places the medulla suffers the most, though in others the cortex shares in the atrophy. The calices on the posterior surface are markedly distended, the anterior ones less so.

The left ureter is lobulated and has somewhat the appearance of a string of sausages. It is bow shaped and on its inner curvature measures 28 cm., on its outer, 34 cm. It is markedly dilated and varies from 3 to 4 cm. in diameter, and is nearly filled with about 200 c.c. of clear, amber-colored liquid which flows freely from one part to another. The ureter does not communicate with the bladder, although it extends to it. On section, the wall is thin, and valve-like folds are seen which cause narrowing of the lumen in places. The mucosa throughout is normal in appearance. The left pelvis is slightly enlarged and envelopes about two-thirds of the circumference of the kidney. It communicates only with a single calyx. The capsule of the left kidney is markedly adherent, but can be stripped off on one side. The surface is irregular and granular. A

few small cysts are scattered about. The consistency is markedly increased. The organ measures 2 cm. long, 8 mm. deep and 1 cm. thick. On section, it is very pale and very dense. The cortex is with difficulty differentiated from the medulla.

Histological Examination.—Right kidney. Sections from the thinner portions show rather distinct differences from those of the thicker parts. In the former, the glomeruli are either hyalin or fibroid. The tubules are few, shrunken, degenerating and show marked atrophy. Hyalin casts fill the lumina of some. There is a great increase of fibrous tissue with marked round cell infiltration. The arteries show thickening of the intima and many are obliterated. The pelvic lining shows marked fibrino-purulent inflammation, in which long slender bacteria are seen. In the thicker portion numerous glomeruli are increased in size, many of them being considerably larger than normal. Many, however, show fibrous thickening of the capsule with hyalin and fibrous changes in the tufts and so merge into the character of those described in the thinner parts. The tubules are apparently larger than normal, and the epithelial cells are generally swollen. In places numerous long, slender bacteria are seen in the lumina. In some places the interstitial tissue shows no change, while in others, there is marked increase with areas infiltrated with leucocytes. In these areas most of the leucocytes are polymorphonuclear neutrophiles, though there are many plasma cells and a few eosinophiles. Scattered throughout this portion of the kidney are larger and smaller abscesses, mostly in the medulla, causing necrosis of tissue with marked leucocytic infiltration. The arteries show no marked changes. There are a few small hæmorrhages. The pelvic lining shows an acute inflammatory process.

Left kidney. The sections consist chiefly of rather dense, fibrous tissue containing numerous nuclei. The cortex is very irregular. Apparently all the glomeruli are undergoing fibrosis, which is more or less marked, and many are replaced by a hyalin mass. The tubules are much reduced in number, and show various changes. A few are lined with a single row of cuboidal cells. Others are small and shrunken, with the lumina filled with granular detritus. Still others show varying degrees of dilatation up to cystic cavities which are from 50 to 100 mm. in diameter. Some of the tubules contain hyalin, and others colloid-like material. The interstitial tissue is everywhere greatly increased and in many places there is considerable round cell infiltration.

Cultures were made from the right kidney and bladder and from the right ureter. From the two former locations Bacillus coli communis was obtained; the culture from the right ureter remained sterile.

THE ELECTRICAL CHARGE OF TOXIN AND ANTITOXIN.

By CYRUS W. FIELD AND OSCAR TEAGUE.

(From the Research Laboratory of the Department of Health, New York City.)

Soon after the discovery of diphtheria antitoxin, several investigators attempted to convert diphtheria toxin into antitoxin by the electrical current, and some went so far as to suppose that this method would supersede the costly and time-consuming process of immunizing animals. Smirnow[1] inoculated rabbits with half a cubic centimeter of a two to three days old broth culture of diphtheria bacilli, and twenty-four hours later, when the animals were sick, injected 10 cubic centimeters of the anodal fluid, obtained by passing a current for eighteen hours through diphtheria toxin. According to him, the animals were saved by the injections. Bolton and Pease[2] stated that two cubic centimeters of the anodal fluid obtained from diphtheria toxin neutralized ten minimal lethal doses of the toxin. It is a well-established fact that acids destroy diphtheria toxin more readily than alkalies, and hence it is to be considered that it was the acid at anode which in Bolton and Pease's experiments neutralized the toxin. The latter investigators believed that the electric current caused a rearrangement of the constituent atoms of the toxin molecule, so that antitoxin resulted; but they did not determine whether the toxin molecule moved with or against the current by virtue of the charge which it carried.

The first to undertake the determination of the electro-positive or electro-negative nature of diphtheria toxin and antitoxin was Römer.[3] Römer used a U-shaped tube and allowed the electrodes to dip into the toxin and antitoxin to be investigated. After the current had been passed for a stated interval of time, the fluid was pipetted from both branches of the tube simultaneously and tested

[1] Smirnow, *Berl. klin. Woch.*, 1892, xxxii, 645.

[2] Bolton and Pease, *Jour. of Exper. Med.*, 1896, i, 537.

[3] Romer, *Berl. klin. Woch.*, 1904, xli, 209.

on guinea-pigs for toxic or antitoxic properties. The results were entirely negative, since he was unable to determine whether the toxin or antitoxin particles traveled toward the cathode or anode. This failure was due primarily, we believe, to the destruction of the toxin and antitoxin by the products of electrolysis, brought about by the strong current employed, for Römer states that he used oil to get rid of the bubbles at the electrodes. Furthermore, the method of simultaneous pipetting very likely caused some mixing of the different portions of the fluid.

To avoid these errors in technique and to eliminate the effects of electrolytic products, we first used a modification of Bilitzer's cells, as shown in Diagram 1. The cells and connecting tubes were of

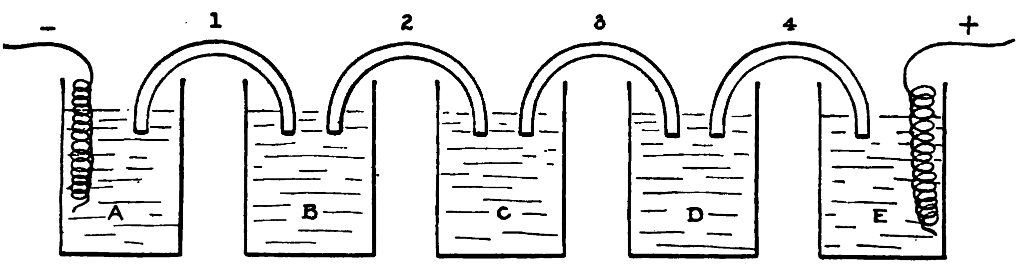

Fig. 1.

glass; the electrodes consisted of coils of platinum wire. The cells *A, B, D* and *E* were filled with distilled water, which was brought to the same level in all of them by means of water-filled connecting tubes. The central cell, *C,* was then filled to a slightly lower level than the others with the toxin or antitoxin to be tested. The small connecting tubes *1* and *4* being in place, and the direct lighting current turned on, the tubes *2* and *3* were filled with distilled water and simultaneously placed in position, thus completing the circuit. After the current had been passed for the desired length of time, the connecting tubes *2* and *3* were removed at the same moment, care being taken that they remained filled. Then *1* and *4* were removed. Under the influence of the current, the fluid in *A* became alkaline, that in *E,* acid, while *B* and *D* remained almost neutral. Hence, only the fluid in the two latter cells was tested on guinea-pigs for toxic or antitoxic value. The results

were unsatisfactory and contradictory, probably owing to the fact that some of the test fluid passed into the neighboring cells along the outside surface of the connecting tubes, being drawn up by capillarity. When we used longer connecting tubes, the internal resistance became too great for the passage of an appreciable amount of current. This method was therefore discarded.

The following apparatus yielded decisive results. Two semi-circular glass tubes, each 1 cm. in diameter and 20 cm. long, were filled with melted agar (2 per cent. agar in distilled water) and allowed to cool to the temperature of the room. These were then arranged as in Diagram 2, the same platinum electrodes being used as in the previous experiments. The toxin or antitoxin to be tested was placed in the middle beaker, distilled water, in the other two.

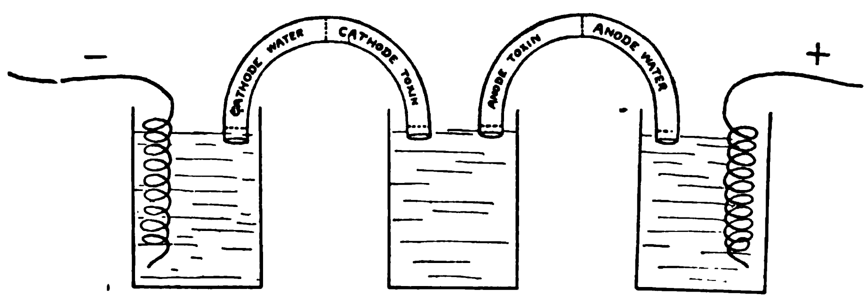

FIG. 2.

The direct Edison street lighting current, 110 volts, was passed for from four to five hours,[4] the distilled water in the end beakers being siphoned off and renewed every half hour to eliminate the disturbing influence of the products of electrolysis. The agar tubes were now removed and the ends thoroughly rinsed in distilled water. The agar mass was then forced from that portion of the tube which dipped into the toxin or antitoxin out through the other end. About a quarter of an inch was removed from each end of the mass, and the remainder divided approximately in half. Each

[4] The current was passed for only four or five hours because the risk of a disturbance due to products of electrolysis increased with the time; moreover, Bredig, Hardy, Pauli, and others found that the passage of a current for twenty-four hours or more would often cause a reversal of the charge carried by particles which would then necessarily be driven back in the opposite direction, thus obscuring the nature of the charge which they originally carried.

of these portions was rinsed in distilled water, chopped into fine pieces, and allowed to stand for one hour in about 6 c.c. of distilled water. At the end of this time the agar was removed by filtering through gauze, and the filtrate was tested on guinea-pigs

TABLE I.

Substance Tested by Passage of Electric Current.	Acidity or Alkalinity.	Cathode Water.	Cathode Toxin or Antitoxin.	Anode Toxin or Antitoxin.	Anode Water.
Diphtheria toxin.	Acid. Alkaline.	No reaction. No reaction.	+ in 20 hours. + in 28 hours.	No reaction. No reaction.	No reaction. No reaction.
Tetanus toxin.	Acid. Alkaline.	No reaction. No reaction.	+ in 48 hours. + in 40 hours.	No reaction. No reaction.	No reaction. No reaction.
Diphtheria antitoxic serum. Tested against 35 M. L. D.'s.	Acid. Alkaline.	Protected. Protected.	Protected. Protected.	+ in 22 hours. + in 42 hours.	+ in 24 hours. + in 38 hours.
Diphtheria antitoxic globulins.[5] Tested against 35 M. L. D.'s.	Acid. Alkaline.	+ in 36 hours. + in 24 hours.	Protected. Protected.	+ in 36 hours. + in 36 hours.	+ in 36 hours. + in 36 hours.
Tetanus antitoxic serum. Tested against 35 M. L. D.'s.	Acid. Alkaline.	Protected. Protected.	Protected. Protected.	+ in 50 hours. + in 48 hours.	+ in 68 hours. + in 48 hours.
Normal broth. No toxin.	Acid. Alkaline.	No reaction. No reaction.	No reaction. No reaction.	No reaction. No reaction.	No reaction. No reaction.
Normal horse serum. No antitoxin. Tested against 35 M. L. D.'s.	Acid. Alkaline.	+ in 36 hours. + in 40 hours.	+ in 36 hours. + in 42 hours.	+ in 36 hours. + in 40 hours.	+ in 36 hours. + in 40 hours.

Test without the electric current. Diphtheria toxin. Diphtheria antitoxin.[5] Tested against 35 M. L. D.'s.	Water half negative + in 40 hours.	Toxin half negative + in 41 hours.

The first .5 cm. of the agar dipping into the toxin or antitoxin was removed, as was always done in our experiments with the electric current. There was always a slight trace of toxin or antitoxin in the first half centimeter, but it was never found beyond this point. A similar phenomenon was observed by Flexner and Noguchi, in relation to the diffusion of tetanus toxin into agar.

for toxic or antitoxic properties. The accompanying table gives the results of these experiments.

[5] Gibson, *Jour. of Biol. Chem.*, 1905, i, 161; Flexner and Noguchi, *Jour. of Exper. Med.*, 1906, viii, 547.

In order that the table may be more readily understood, a detailed explanation of one of the experiments (No. 6, for example) contained in it may not be superfluous.

Diphtheria antitoxic serum was made slightly alkaline to phenolphthalein by the addition of a small amount of 0.1 normal sodium hydrate. The agar tubes were placed in position, as in Diagram 2, and the current was passed through it for four hours. The four portions of agar, which we shall designate as cathode water, cathode-antitoxin, anode water, and anode-antitoxin, were then cut into fine pieces and extracted in water for one hour, and to each of these extracts was added 35 m. l. d. of diphtheria toxin. These four portions of fluid were now injected subcutaneously into the adbominal walls of four guinea-pigs each weighing about 250 grams.

The two guinea-pigs receiving the fluid from the anode water and anode-antitoxin portions of agar died in thirty-eight and forty-two hours, respectively; hence, these portions must have contained no antitoxin. Those receiving the fluid from the cathode water and cathode-antitoxin portions showed no induration at the point of injection and no loss of weight for five days, after which they were discharged. Hence, they were fully protected against the 35 m. l. d. of toxin.

The table shows that under the influence of an electric current, the particles of both toxin and antitoxin travel toward the cathode, and that a change in the reaction of the solvent does not cause a reversal of the charge carried by the particles.

Blitz, Much and Siebert[6] claim that both tetanus toxin and tetanus antitoxin are precipitated by electro-positive inorganic colloids and conclude, therefore, that they are electro-negative and should, under the influence of an electric current, pass toward the anode. However, neither they nor Römer were able to demonstrate this fact experimentally. It is probable that the precipitates which they obtained were due either to the action of electrolytes contained in the toxin or antitoxin on their inorganic colloids, or to the action of these colloids on non-toxic or non-antitoxic protein substances, or to a combination of both. It is worthy of note that their non-

[6] Blitz, Much and Siebert, *Beit. zu exper. Therapie,* 1905.

toxic broth gave precipitates with practically the same inorganic colloids as did the toxin.

Hardy[7] states that proteins are amphoteric; that is, that in an acid medium they travel toward the cathode and in an alkaline medium toward the anode, while in a neutral medium they do not move toward either pole. He worked with an albumen coagulated by heat. Pauli,[8] using a protein solution obtained by dialyzing serum from eight to ten weeks and filtering off the euglobulin, found, in agreement with Hardy, that the protein was electro-positive in an acid solution and electro-negative in an alkaline one.[9] We have shown that alteration of the reaction of the solvent does not change the character of the charge carried by particles of toxin or antitoxin. If proteins are amphoteric, as is generally believed, then this observation of ours would point to the non-protein nature of toxin and antitoxin. In one series of experiments, however, we have found that the portion of agar containing the toxin or antitoxin, that is, the cathode portion, was the only one to give the biuret reaction and this occurred when the test substance was in either an acid or an alkaline solution. If experiments which we have under way should verify the last results, it would indicate that native proteins may not be amphoteric and then the argument that we have advanced as to the non-protein nature of toxin and antitoxin would be invalidated.[10]

If the combination of toxin with antitoxin is a true chemical reaction, one would expect that under the influence of an electric current toxin would travel in one direction and antitoxin in the opposite direction. Such, however, was not found to be the case and we are, therefore, inclined to believe that this union is not a true chemical reaction, but a matter of adsorption, as was first suggested by Bordet, and has since been claimed by others.

[7] Hardy, *Jour. of Physiology*, 1899, xxiv, 288.

[8] Pauli, *Hofmeister's Beit.*, 1906, vii, 531.

[9] Pauli denaturalized his proteids by the prolonged dialysis.

[10] Oppenheimer (Toxin and Antitoxin, 1903) in summing up the work on this subject concluded that toxin and antitoxins are of non-protein nature. Quite recently Osborn, Mendel and Harris (*Amer. Jour. Physiol.*, 1905, xiv, 259), working with ricin, have taken issue with him, having found that their purest product still gave protein reactions. As a matter of fact, nothing definite is known at present of the chemical nature of these various substances.

CONCLUSIONS.

1. Both diphtheria and tetanus toxin and their antitoxins are electro-positive, that is, they pass to the cathode under the influence of an electric current.

2. The character of the charge is not altered by a change in the reaction of the solvent.

3. The combination of toxin and antitoxin would seem to represent not a true chemical reaction but the adsorption of one colloid by another.

AN EXPERIMENTAL STUDY INTO THE CAUSE OF THE INCREASED PORTAL PRESSURE IN PORTAL CIRRHOSIS.[1]

By FREDERICK C. HERRICK, Cleveland.

I.

In reviewing the subject of portal cirrhosis, it is evident that our understanding of the cause, course and pathological physiology of the disease is far from clear. The pathological anatomy is fortunately well understood and one need but to refer to the recent compilation by Dr. Rolleston to find a clear description and statistics of every phase of the process. But when we enter the domain of the pathological physiology of the disease not one of its chief departures from the normal physiology is satisfactorily explained.

The increase in the portal blood pressure is one of the most marked abnormal conditions developed. As evidence that this condition exists we have the frequent hæmorrhages, the collateral circulation and the ascites, by many considered as a result of increased portal pressure. The fact of this increase in pressure cannot be doubted, but the cause of this condition, heretofore considered to be the obstruction to the portal vessels by the fibrosis and its contraction, is, in the light of the following observations, open to question.

The object of this research was to explain the increased portal pressure in this disease and thus to throw further light on the collateral circulations frequently developed naturally as well as following the operation of Talma and similar operations. In order to gain this end the circulation of the normal liver was first studied.

A short historical resume may not be out of place.

[1] The following experimental work was done in the Pathological Laboratory of the London Hospital, London, England. I am greatly indebted to Dr. W. C. Miller, pathologist to the hospital, for this privilege and also for the material which was necessary for the work.

Betz[2] in 1863 worked in Ludwig's laboratory on the question: Does the amount of blood entering the liver through the two vessels depend on their relative pressures? If this is so he recognized that the arterial flow if of large enough volume would exclude the portal. He injected many livers of dogs with hardening fluid and measured the corresponding arteries and veins, finding their proportion in size to be as 1:5 though there were wide variations. Large arterial trunks were frequently near relatively small portal vessels. The relative volume flow between the hepatic artery and the portal vein he found to be as 1: from 2 to 4 when the two vessels were flowing at different times. When flowing during the same time, they were as 1:50. His arterial pressure was twice the portal which is of course much below the normal ratio (1: from 13 to 15). Still this shows the influence of the portal pressure in decreasing the arterial flow.

Gad[3] in 1873 wrote a dissertation based upon experimental work in which he concluded that the arterial circulation in the liver performed a double function in that it brought oxygen and mechanically controlled the portal flow. A further point suggested by Gad was the aspirating action of the arterial on the portal current as the former passed by the openings of the latter, joining with them at an acute angle. He further found by experiments with rabbit's livers that a current through the arteries retarded the portal flow.

To this observation may be added the fact that the hepatic artery is capable of bringing the pre-urea bodies to the liver for their formation into urea and even in health this occurs to a certain extent.[4] At least four cases are on record in which the portal vein was entirely obliterated and the individuals lived for years; two were congenital malformations.[5] Dogs with Eck fistulas and with their portals tied excrete a normal amount of urea and have been kept for months in good health. It is therefore certain that the arterial circulation has for its functions (1) the nourishment of the connective tissue of the liver, (2) an influence on the amount of blood passing through the portal vessels, this influence being a factor in the circulatory balance within the liver, (3) the power to carry sufficient blood to the liver lobule to ensure a normal excretion of urea and an activity of the liver function sufficient to maintain health.

II.

Within the normal liver there are six factors to be considered.

1. A large volume-circulation with a low pressure.
2. A small volume-circulation with a high pressure.
3. A common channel of exit for fluid entering by these two circulations.

[2] Betz, *Zeit. für rat. Med.*, 1863, xviii, 44.

[3] Gad, Dissertation, Berlin, 1873.

[4] Hahn, Massen, Nencki, Pawlow, *Arch. f. exper. Path. und Pharm.*, 1893, xxxii, 161. Herrick, *Jour. of Exper. Med.*, 1905, vii, 751.

[5] Abernethy, *Philosophical Trans.*, 793, p. 61; Kierman, *Philosophical Trans.*, 1833, p. 758; Lawrence, *Medico-Chirurg. Trans.*, 1814, v, 174; Osler, Practice of Med., 1905.

4. A freely expansible tissue framework.

5. Two methods by which the entering circulations may influence each other, *i. e.*, by direct communication or by lateral pressure.

6. A vaso-motor mechanism to both sets of vessels.

Considering the large volume-circulation, Quincke (5) quotes von Basch who estimated the portal pressure when the splanchnic nerve was cut in a dog, at from 7 to 16 mm. of mercury, and Heidenhain who found it from 5.2 to 7.2 mm. without nerve section. The volume-flow of the portal vein was estimated by Cybulski (5) in a "small dog" at from 2.4 to 2.7 c.c. per second (a proportionate estimate in a man of 70 kilos would give the portal flow as about 35 c.c. per second). I have not been able to find a record of estimates of the volume-flow of the hepatic artery or its blood pressure. So far as size is concerned it may be considered as at least equal to the brachial artery, which has a pressure of 130 mm.

The freely expansible tissue framework is a most important factor towards the maintaining of the proper balance between the two circulations. This point and the next one will be discussed later when the experimental data have been given. Schäfer[6] concludes that both the intrahepatic arterial and portal vessels possess vaso-motor constricting powers. His evidence is not entirely convincing. A study of this influence, however, has not been included in this research, the aim being to draw some conclusions regarding the capacities of the intrahepatic veins and arteries, considering them as so many anastomosing, more or less expansive tubules lying adjacent to each other in a framework of varying expansibility.

That the widely differing pressures of the portal vein and hepatic artery at the porta hepatis come to a common level at the junction of their inter-lobular or intra-lobular venules is certain. This equalization comes about through the medium of the following factors:

1. A direct communication exists between the veins returning from the arterial supply and the portal venules. Regarding this I quote from Landois.[7] "The branches of the hepatic artery

[6] Schäfer, Text-book of Physiology, 1900, 140, 643.

[7] Landois, Text-book of Physiology. p. 308.

throughout their entire course accompany the larger branches of the portal vein (to which as well as to the adjacent larger bile ducts they supply nutrient capillaries). These branches enter into numerous anastomotic communications among themselves. The small capillaries pass mainly from the periphery of the acinus into the capillaries, however, that lie in the thicker connective tissue upon the larger venous and biliary branches and pass over chiefly into two venous trunks that, accompanying the corresponding arterial branches for some distance, empty into branches of the portal vein. Individual arterial branches pass up to the surface of the liver, where they form a wide nutritive network, particularly under the peritoneal covering. The small venous radicles collecting from this point also reach the ramifications of the portal vein."

The arterial supply thus passes through a capillary system arranged in Glisson's capsule about the portal interlobular veins and its pressure becomes reduced to an ordinary venous one. This arterial division has also been described as resulting in the formation of three or four arterioles which wind spirally about their accompanying portal vein. This spiral arrangement can be demonstrated in microscopical sections.

2. The vaso-motor influence in balancing these flows must be considered. Thus it has been shown by Bayliss and Starling that a stimulation of the splanchnic nerves causes an increased flow of blood from the liver, although the portal vein was tied. It has also been shown by Mall[8] that the portal vein receives fibers from the splanchnics. In my work in making the Eck fistula, I have frequently noticed slow vermicular contractions of the portal vein following mechanical stimulation of its walls. Two Italian authors ascribe both constrictor and dilator fibers to the splanchnic nerves and dilator fibers to the vagus (Cavazzani and Manca[9]).

Thus, in the normal liver through the medium of these two factors the arterial and portal pressures reach a common level at the points where their currents join to form the intralobular veins. If the arterial supply to a given area is relatively increased a disturbance of the balance will occur. Also, a fibrosis will make the vessel

[8] Mall, *Arch. f. Physiol.*, 1892, 409 (from Schäfer).

[9] Cavazzani and Manca, *Arch. ital. de biol.*, 1895, xxiv, 35, 295.

walls more rigid, less variable in caliber and if this intra-hepatic balance is partly due to a vaso-motor influence, this must be altered by an advancing fibrosis. I therefore sought to determine the influence on each other as to pressure and volume-flow of changes in the portal and arterial pressures and to measure the volume-flow of the two currents entering the liver at pressures considered as normal. I next made the same observations upon cirrhotic livers and found a marked difference, as will be shown.

III.

The methods employed were as follows:

For normal livers only those were used which were free from possible disease. The history and pathological diagnosis will be given. No livers altered by passive congestion were used; only typical cirrhotic livers were used for that part of the research which concerns this condition. Microscopical sections were made of each liver and the condition verified. The livers were taken never longer than twenty-four hours post mortem and were never kept longer than twenty-four hours after being removed from the body. When they were kept over night it was always in normal saline in cold storage. Comparisons of the two circulations in the *same* liver only were made. The two circulations in different livers were not compared. The circulating fluids used were normal saline solution at 38–39° C. At the end of the experiments I used defibrinated sheep's blood fresh from the animals for verification. The livers were always immersed in their natural position in normal saline at body temperature. Mercury manometers were used to measure pressures. In every case the liver was washed of blood at the normal pressures of 130 mm. for the arterial and 10 mm. for the portal vessels. They were next washed backward by a pressure of 10 to 20 mm. through the hepatic veins. It required from ten to fifteen minutes to wash a liver preparatory to the experiments. Bits of washed fibrin were occasionally floated out and especially backward from the hepatic to the portal vein. The first estimations were always made on both circulations together at normal pressures. These were then varied within the limits of from zero to 140 mm. arterial, and zero to 20 mm. portal pressure. The pressures were always put on together, and variations made as nearly as possible in the normal ratio. The portal circulation was next studied alone at variable pressures. The arterial was then considered alone with its higher pressures. By this order an unnatural dilatation of the vessels was prevented.

After the experiments the livers were sliced into sections one centimeter thick and the macroscopic vessels examined for possible occluding fibrin. In but one case was fibrin found in a portal vessel of importance, a vein of about three millimeters diameter. Sections for histological study were then made.

IV.

The effects on the portal pressure of changes in the arterial pressure and vice versa were first studied.

Arterial Pressure.	Portal Pressure, Normal Liver.	Portal Pressure, Cirrhotic Liver.
0	10	10
30	10	15
50	10	18
100	11	20
130	12	30
150	14	35

Portal Pressure.	Arterial Pressure, Normal Liver.	Arterial Pressure, Cirrhotic Liver.
5	130	130
30	130	135
40	135	140
10	127 (?)	133

In normal livers an increase in the arterial pressure, when the portal was flowing at normal pressure of 10 mm., caused a very slight rise in the portal pressure, *i. e.,* about 1 mm. rise of portal for every 40 mm. rise of arterial pressure. In making this observation the portal was first set at 10 mm. and, when flowing evenly, the arterial flow was gradually turned on. Thus, raising the arterial pressure from zero to 130 mm. caused a rise of but 3 or 4 mm. in the portal pressure. When this same observation was made on a liver with marked portal cirrhosis a great difference was noted. In this case a rise of 130 mm. in the arterial pressure caused a rise of from 20 to 30 mm. in the portal pressure.

With the normal liver the rise of *portal* pressure was 1 mm. for every 40 mm. of arterial pressure increase; with the cirrhotic liver it was 1 mm. for every 6 mm. of arterial pressure increase.

When in a normal liver the *arterial* pressure was first set at 130 mm. and the portal gradually opened a similar result was noted. The arterial rise was 1 mm. for every 7 mm. increase in portal pressure.

A similar observation in a cirrhotic liver was obviously impossible, for so soon as the arterial pressure was set at 130 mm. the portal pressure rose immediately to from 20 to 25 mm., although the portal flow had not been opened, and this occurred, though more slowly, regardless of whether the portal vessels were ***full***

or not. If they had been drained *before* this observation the fluid flowed into them, as will be shown in the next observation, filled them and registered the pressure.

After this arterial pressure was thus set and the portal pressure was stationary at say 25 mm., a further rise in portal pressure, caused by turning on its flow, was followed by a rise in arterial pressure of 1 mm. for every 3 mm. increase in portal pressure. Thus in the normal liver the *arterial* pressure rose 1 mm. for every 7 mm. increase in portal pressure; in the cirrhotic liver it rose 1 mm. for every 3 mm. increase in portal pressure.

It will be seen that arterial rise in pressure did not affect the portal pressure in normal livers until it had passed 100 mm. I did not measure the effects of pressures above 150 mm. In cirrhotic livers the effect on the portal pressure was evident at 30 mm. arterial pressure. These effects of one pressure on the other occurred immediately on changing a pressure and show as clearly as possible the mutual influence between the portal and arterial pressures within the liver, and is, I believe, an important explanation of the rise in portal pressure in portal cirrhosis.

As an explanation of this marked effect on each other in portal cirrhosis I may offer the following suggestions:

1. The arterial supply to the fibrous tissue of a cirrhotic liver is increased. Thus an increased amount of fluid passes through the arterial capillaries, enters the portal venules and raises the portal pressure there.

2. This would be especially the case if the hepatic veins were not quite able to give passage to the increased volume of fluid. (During life a slight weakening of the heart muscle might favor this backward pressure.)

3. There was a freer flow of fluid through the arterial capillaries into the portal veins, as will be shown later. This could occur by a dilation of these capillaries which would favor a greater influence of the arterial on the portal pressure.

V.

The arterial and portal pressures in the normal liver are perfectly balanced at the point where the arterial return veins enter

the portal venules. In the liver of portal cirrhosis this balance is disturbed by (1) the larger arterial supply, (2) the loss of portal influence by lateral pressure on the arterial capillaries and the freer communication of the arterial with the portal vessels by means of dilated capillaries. This free anastomosis between the arterial and portal vessels within the liver has been shown above (Landois). As further evidence of these communications I wish to cite the following observation:

The arterial flow alone was connected, leaving the portal and hepatic veins with a glass cannula tied in each for collecting the fluid returning through them. There was always a free return flow through both veins from an arterial inflow. Further, in the normal liver, the portal return flow was less in volume than that from the hepatic vein. In the markedly portal cirrhotic liver the portal return flow was always greater than that from the hepatic vein. This is what we would expect from the previous observations on pressure.

When the inflow was through the *portal* vein and the hepatic vein and artery were left open the return flow was *entirely* through the hepatic vein in both normal and cirrhotic livers. No fluid returned through the hepatic artery. This observation may be explained in one of the following ways:

1. Gad described the arterial return venules as uniting with the portal at an acute angle leaving a wedge-shaped valve between them. With a like pressure in the portal veins and these entering venules there would be a free flow through both; but if the pressure of either was increased there would be a narrowing of the other's lumen and reduction in its flow. If there is no arterial flow it is evident the large portal volume will entirely occlude the entering veins from the arterial capillaries, and fluid cannot flow back through them, as seen above. When, on the other hand, there is no portal flow and there is a flow through the arteries, their channels being of much less volume than the portal veins, are manifestly unable to occlude the portal vessels and hence the fluid returns through them.

2. The fact that no fluid returned through the hepatic artery from a portal inflow may also be explained by the intralobular capil-

laries and hepatic vein offering less resistance than the arterial capillaries. Against this, however, is the fact that in the cirrhotic livers where the arterial capillaries are dilated no fluid returned through them and the hepatic artery.

3. Any obstruction in the hepatic veins might cause this regurgitation of fluid through the portals. I cannot find any evidence of such obstruction. There was no intra-lobular fibrosis in these livers and, from the experimental side, there were no occluding blood clots. We must, therefore, conclude that this is not an explanation of the above facts. The wedge-shaped valves of Gad, together with the difficulty of fluids regurgitating through a capillary network, must explain the fact that an inflow through the portal returns entirely through the hepatic veins.

VI.

The volume of flow from the portal and arterial vessels is the subject of the next observations to which I wish to draw attention. Certain proportions were evident, as shown in the following table. The time during which each flow was measured was one minute.

	Volume flow of portal vein.			
		10 mm.	20 mm.	30 mm.
Normal	45 oz.	230 c.c.	313 c.c.	
	52 oz.	422 c.c.	662 c.c.	
Cirrhotic	68 oz.	758 c.c.	1144 c.c	
	128 oz.	880 c.c.	1230 c.c.	2100 c.c.

Vol. flow of portal vein in cirrhosis calculated proportionately to weight of normal livers.	
10 mm.	20 mm.
668 c.c.	996 c.c.
Actual vol. flow.	
819 c.c.	1187 c.c.

From this table it will be seen that the average volume flow of the portal vein in the cirrhotic livers was, in the proportion to their weights, greater than that of the normal livers. In a normal liver weighing 45 oz. the flow from the portal vein alone was 230 c.c. in one minute at 10 mm. pressure; in another weighing 52 oz. the flow was 422 c.c. at the same pressure.

In a cirrhotic liver weighing 68 oz. (portal cirrhosis) with roughened surface and adherent thickened capsule the portal volume flow in one minute at 10 mm. pressure was 758 c.c. In another weighing 128 oz. a typical large "hob nailed" liver with marked peri-portal fibrosis, opaque thickened capsule, many adhesions, the

portal vessel allowed a flow of 880 c.c. in one minute at 10 mm. pressure. Thus the portal vascular capacity in portal cirrhosis, so far from being *decreased,* appears to be proportionately *increased.* These comparisons were verified on other livers as well and it was found that from a cirrhotic liver flowed at the same pressure at least as much fluid proportionately to its weight as from the normal one. This shows that there can be no obstruction to the portal vessels within the liver from fibrous tissue. The portal vessels allowed fluid to pass freely and it was evident as soon as such a liver was transfused that the flow was quite as free as that through a normal liver.

So soon as the arterial flow was added a different state of affairs existed. Now the portal flow was decidedly limited.

With a normal liver weighing 61 oz.

Art. Pres.	Port. Pres.	Time.	Vol.
130 mm.	10 mm.	1 min.	1024 c.c. Art.
			632 c.c. Port.
		Repeated.	1032 c.c. Art.
			650 c.c. Port.
130 mm.	20 mm.	1 min.	1100 c.c. Art.
			1300 c.c. Port.
		Repeated.	1060 c.c. Art.
			1280 c.c. Port.

With a cirrhotic liver weighing 128 oz.

Art. Pres.	Port. Pres.	Time.	Vol.
130 mm.	25 mm.	1 min.	1250 c.c. Art.
			1050 c.c. Port.
130 mm.	30 mm.	1 min.	1750 c.c. Art.
			1250 c.c. Port.
130 mm.	40 mm.	1 min.	1550 c.c. Art.
			2750 c.c. Port.
130 mm.	50 mm.	1 min.	1525 c.c. Art.
			2950 c.c. Port.

Thus at 130 mm. arterial and 10 mm. portal pressure the arterial volume-flow was the larger. It is generally conceded that the portal volume-flow is normally greater. This relation occurred between 10 and 20 mm. portal pressure. In the cirrhotic liver this relation occurred only at a portal pressure between 30 and 40 mm. Up to this point the arterial flow was decidedly preponderant.

In the above observations on a cirrhotic liver the portal pressures are seen to be very high. As shown in Part IV, when such a liver was placed under observation it was found that the influence of one circulation on the other was much more marked than in a normal organ. With an arterial pressure of 130 mm. in the hepatic artery it was found impossible to have a normal portal pressure of 10 mm. The portal pressure immediately went to 20 mm., although the portal flow *had not been opened*. In making the above observations, therefore, the arterial pressure was first set at 130 mm., and after it was flowing evenly and the pressure in the manometer connected with the portal vein had risen to its maximum, the portal flow was opened and regulated so as to add from 5 to 30 mm. more pressure.

In a normal liver, to overcome the arterial resistance to the portal flow the portal pressure had to be raised above 10 mm. At 10 mm. portal pressure and 130 mm. arterial pressure the arterial flow was greater. When the portal pressure was 20 mm. its flow became greater.

In a cirrhotic liver the portal pressure necessary to resist 130 mm. arterial pressure was much higher, being 40 mm. The circulatory balance is maintained in a cirrhotic liver at a portal pressure much above that considered as normal. By circulatory balance I have meant the pressures at which the arterial and portal vessels, when flowing together, allowed the flow of the largest amounts of fluid.

Conclusions.

1. In the liver of portal cirrhosis there is a far freer communication between the arterial and portal currents than in the normal liver.

2. Factors contributing to the increased portal pressure in portal cirrhosis are (1) the direct communication of the arterial pressure to the portal vessels through dilated capillaries, (2) the larger volume-flow of the hepatic artery in proportion to the portal flow in cirrhosis as compared to that in the normal liver.

3. A portal cirrhotic liver gives passage to an amount of portal fluid proportionate to its weight. There is no obstruction to the portal vessels from fibrosis in the large portal cirrhotic liver.

4. From an arterial inflow there is a free return flow through the portal as well as through the hepatic veins in both normal and cirrhotic livers.

5. From a portal inflow the return is through the hepatic vein only. The Gad's theory of valves and the arterial capillary network account for this fact.

6. The portal pressure has a decided influence on the arterial volume-flow and vice versa. This influence is more marked in the cirrhotic than in the normal liver.

7. The communication of the arterial pressure to the portal pressure is an important factor in an explanation of the increased portal pressure in portal cirrhosis.

Autopsy 959. (Year 1905.) Female, 18 yrs., burned on thorax, head, arms; death eighteen hours later. Liver normal, wt. 52 oz. Histological examination showed normal liver tissue.

Autopsy 953. Female, 21 yrs. Perforating gastric ulcer; death twenty hours later from general peritonitis. Liver, normal, wt. 45 oz. Microscopical examination showed a normal liver tissue.

Autopsy 970. Male, 48 yrs., brought in dead. Post mortem diagnosis: fibrosis of mycardium and partial occlusion of the coronary arteries by atheroma. Liver, 61 oz. Capsule thin, normal. Microscopical examination showed normal liver tissue.

Autopsy 998. Female, 60 yrs., general arterio-sclerosis, bronchitis, emphysema; arterio-sclerotic kidneys. Liver wt. 68 oz., surface rough and irregular, diaphragm adherent, capsule thickened, substance of liver brown, fibrous, tough. Microscope showed peri-portal fibrosis.

Autopsy 1055. Male, 44 yrs. Cause of death, injury. Liver, large, tough, pale. Microscopical examination shows rather a marked peri-portal fibrosis, in places isolating the lobules from each other.

Autopsy 1086. Male, 63 yrs. Epithelioma of penis. Liver wt. 128 oz., hob nailed, tawny yellow, tough, capsule opaque and thickened. Many adhesions about porta hepatis, and between the capsule and the diaphragm. Microscopical examination showed a typical, peri-portal fibrosis of marked degree.

CONTRIBUTIONS TO THE BIOLOGY OF DIPLOCOCCUS INTRACELLULARIS.

By SIMON FLEXNER, M.D.

(From the Rockefeller Institute for Medical Research, New York City.)

INTRODUCTION.

Cerebro-spinal meningitis prevailed as an epidemic in Greater New York City during the winter of 1904 and the spring of 1905. The epidemic embraced about 4000 cases of the disease. The deaths numbered 3,429. In the year 1904, 1,403 deaths occurred from meningitis. In the year 1905, the number of cases of the disease reported was 2,755, and the number of deaths, 2,026. The percentage mortality, deduced from the figures for 1905, is 73.5 per cent.

In the spring of 1905, the Health Department of the City of New York appointed a commission to co-operate with the department in investigating the epidemic. As a member of the commission, I undertook an experimental investigation of epidemic meningitis, out of which the following reports have grown. The rather one-sided nature of the studies, which are to be given here, is to be explained, in part, by the allotment of different aspects of the investigation to the several members of the laboratory committee of the commission.[1]

The particular subject of investigation alloted to me was the pathogenic properties of *Diplococcus intracellularis,* and the immunization of animals with a view to obtaining immune sera which might be used for the differentiation of the diplococcus by the agglutination test. In the course of the study of its pathogenic effects, I became impressed with certain biological properties of the *Diplococcus intracellularis* which have not hitherto been recorded.

In a small number of instances only did I isolate the diplococcus

[1] The laboratory committee of the Commission consisted of Drs. W. J. Elser, E. K. Dunham, J. M. Van Cott, Jr., and Simon Flexner.

directly from spinal fluids obtained by lumbar puncture, or from exudates secured at autopsy. I relied for the greater part of the cultures with which I worked upon Dr. E. K. Dunham, Dr. Martha Wollstein, of the Babies Hospital, and Dr. E. P. Bernstein, of Mt. Sinai Hospital. I frequently obtained from Drs. Wollstein and Bernstein cultures in the first and second generation on serum-glucose-agar. Dr. Dunham provided me with many strains of *Diplococcus intracellularis* and a number of diplococci whose properties were not identical with this diplococcus. These latter were obtained, for the most part, from the nose and pharynx. I secured a number of strains of diplococci, which I compared with *Diplococcus intracellularis,* from the nose and throat of healthy persons, and from domestic pets, cats and dogs, owned by persons in whose homes cases of meningitis occurred.[2]

The difficulties of cultivating *Diplococcus intracellularis* were greatly reduced by the employment of sheep serum instead of human serum. For this advantage I am indebted to a suggestion of Dr. W. H. Park, of the Department of Health. Sheep-serum-water, prepared according to Hiss' method, is mixed with a beef infusion agar-agar containing 2 per cent. of glucose. The quantity of sheep-serum water need not exceed $\frac{1}{20}$ to $\frac{1}{10}$ of the volume of the agar. It is added to the sterile and melted agar-agar which is afterwards slanted in test tubes, or allowed to congeal on the expanded surface of 16 ounce Blake bottles. The latter are used for obtaining mass cultures.

The identification of the cultures of *Diplococcus intracellularis,* which I studied, offered no special difficulties. Their origin from

[2] The nasal cavities of the domestic pets were examined by my assistant, Mr. Ward. As a rule, it was necessary to etherize the animals in order to obtain satisfactory "swabs" from the upper nasal mucosa. Several cocci or diplococci decolorizing by Gram's method were obtained but they were readily distinguished from *Diplococcus intracellularis.* Through the kindness of Dr. A. C. Abbott, of Philadelphia, Mr. Ward was enabled to make plate cultures from the upper nasal mucous membrane of fifty inmates of the Philadelphia Hospital. At the time of this investigation meningitis was not epidemic in Philadelphia, and it did not become so afterwards. The inoculated plates were divided with Dr. E. K. Dunham. Neither Dr. Dunham nor Mr. Ward obtained from them any diplococcus which could be confounded with the *intracellularis.* Cocci agreeing with *M. catarrhalis* were, however, obtained from several plates.

the spinal canal of persons suffering from epidemic meningitis was always presumptive evidence of their nature. The diplococci fulfilled the ordinary cultural requirements, and they showed the low degree of viability which has been observed commonly. I quickly learned to lay some stress upon rapidity with which involution occurred in cultures which grew luxuriantly, and, later, I learned to trust the fermentative reactions upon sugars of the diplococcus. Dextrose and maltose are feebly attacked by the diplococcus, and acid is produced. Gas is never liberated, and in a sheep-serum-water-litmus medium, containing the sugars mentioned, reddening, but no coagulation, is produced.[3] Certain other diplococci, not staining by Gram's method, with which *Diplococcus intracellularis* might possible be confused, were either devoid of power to ferment sugars, or they possessed wider or more vigorous fermentative power than the intracellularis.[4]

AUTOLYSIS OF THE DIPLOCOCCUS.

The brief vitality of many cultures of the intracellularis is a point of differential importance. Many strains, grown on a favorable medium, unless transplanted to a fresh medium, do not survive beyond two or three days. Indeed, these transplantations do not succeed, frequently, unless considerable quantities of the growth are carried to the fresh tubes, an indication that only a fraction of the cocci have survived, or are still capable of multiplication. Vigorous fresh cultures of 16 to 24 hours' growth stain sharply and uniformly. On the second day, the cocci show irregularities in staining and size, which irregularities are increased by dessication of the surface of the medium and by other injurious causes. Cultures three days old show marked degenerations, and these increase rapidly with age until, at the end of five or six days, or even earlier, no normal cocci persist. Even with very careful preservation, the great majority of the cocci do not survive beyond this period. A few cocci, in carefully preserved cultures of certain strains, may survive for many

[3] The sugar reactions of the diplococcus have been studied especially by Dr. Dunham and Dr. Elser. (E. K. Dunham, *Jour. Infec. Diseases*, 1906, Supplement 2, p. 10; W. J. Elser, *Jour. Med. Research*, 1906, ix, 89.)

[4] The value and limitation of the agglutination reaction are discussed by Dunham in his report, *Op. cit.*

months. The appearance of old cultures is characteristic. At the beginning of the degeneration, swollen, deeply-stained cocci occur among the smaller but usual forms, which take the stain less intensely than fresh cultures. At this stage, tetradic groups appear, especially among the swollen cocci. As degeneration progresses, loss of staining and disintegration ensue, until, finally, staining power is entirely lost and a formless detritus remains.

The slight viability of *Diplococcus intracellularis* when grown outside the body, and the characteristic changes associated with its dissolution, led me to try the effect of a number of experimental conditions upon these phenomena. The low degree of viability was conceivably due to rapid exhaustion of the medium of growth, at least of that superficial layer of the solid medium upon which growth takes place, or of accumulation of growth products, or of these two factors acting together. The possibility must be kept in mind that the life-history of the diplococcus, within and without the body, may be, at best, a brief one. In this case vigorous-looking cocci would appear in cultures only so long as multiplication was proceeding; and once the cocci have reached the expiration of their brief life-period, disintegrative or involutional changes would set in quickly. Some notion of the influence of exhaustion of the medium and accumulation of metabolic and disintegration products upon multiplication and involution, could be obtained by experiment.

A series of tubes containing the glucose-sheep-serum agar was inoculated with the diplococcus by smearing the slanted surfaces with a recent culture. After twenty-four hours' growth at 37° C., the surfaces were washed with successive quantities of sterile salt solution. Some of the tubes were now returned to the thermostat, and others were returned after the surfaces had been covered with a layer of sheep-serum water and then drained free of the excess. Previous tests had shown me that the diplococcus does not grow in sheep-serum water to which glucose has not been added. Examination of the cultures twenty-four to forty-eight hours later showed that growth had taken place in all the tubes, was less abundant than in the first instance, and presented a filmy, granular appearance. Microscopically, typical diplococci were present; at the expiration of forty-eight hours in the thermostat, the number of involution-forms was large.

Owing to the ease and rapidity with which the diplococcus can be made to undergo dissolution (see below), its soluble disintegration products are readily obtained. These products were tested for their effect upon the growth and vitality of the diplococci.

The large flat surface of medium in a one-pint Blake bottle was inoculated with a culture (654) of *Diplococcus intracellularis.* An abundant growth was obtained in twenty-four hours. About 15 c.c. of sterile 0.85 per cent. salt-solution were poured over the surface, and a few drops of toluol added. The bottle was returned to the thermostat until the next day, at which time an almost clear fluid, devoid of toluol, remained overlying the agar surface from which the growth had disappeared. Cover-glass preparations showed disintegration of all the cocci. The fluid was centrifugalized until clear, and distributed to melted glucose agar as follows: each tube contained 8 c.c. of the melted agar (cooled to 60° C.) to which were added 0.5 c.c., 0.8 c.c., 1.0 c.c., 1.5 c.c. and 2.0 c.c., respectively, of the "extract" of the cocci. The tubes were allowed to cool in the slanting position and each was inoculated with a vigorous culture (656) of the diplococcus. The tabulation shows the result. First observation after twenty-four hours.

Quantity of Extract.	Visible Growth.	Microscopical Examination.	Subsequent History.
0.5 c.c.	Moderate.	Pale staining, relatively large, Gram neg. diplococci.	Spread over surface.
0.8 c.c.	None.		Re-inoculated with fresh culture.
1.0 c.c.	Moderate.	Pale staining, relatively large, Gram neg. diplococci.	Spread over surface.
1.5 c.c.	None.		Re-inoculated.
2.0 c.c.	None.		Re-inoculated.

The second observation was made at the expiration of a second twenty-four hours. The spread colonies now yielded an abundant growth; the re-inoculated tubes all gave growths in separate colonies which, on microscopical examination, showed vigorous cocci except in case of the tube receiving 2 c.c. of the "extract" in which the dozen colonies of diplococci presented a swollen and involutional appearance. At the lapse of another twenty-four hours, all the cultures had begun to degenerate or showed more advanced degeneration, the latter being most marked in the tube with 2 c.c. of the "extract." Transplantations to fresh serum-agar surfaces gave growths in all instances.

From these observations, it would appear as if the brief vitality of the cultures grown outside the body is not to be ascribed directly to the exhaustion of the medium of growth, or the accumulation of injurious products of growth, in the short period of twenty-four to forty-eight hours. It may be safely stated that the quantity of disintegration products added to the tubes in the form of "extract" greatly exceeded the possible accumulation under ordinary conditions of cultivation. The higher concentrations of the "extract" undoubtedly inhibited the growth, and exerted an harmful influ-

ence on the morphological appearances of the diplococci. But as the cultures could still be transplanted successfully at the expiration of forty-eight to seventy-two hours, at which period the experiment was terminated, they may be taken to have retained their vitality quite unimpaired.

It has been observed repeatedly that cultures of *Diplococcus intracellularis* tend to survive longer when kept constantly at the thermostat temperature than when kept at varying room temperatures, or in the ice box. That cultures tend to die quickly when placed in the ice box was noted accidentally by me early in this investigation. A series of tests was carried on in order to determine whether any differences could be noted depending, first, upon the concentration of the suspension of cocci kept on ice, and next, upon intermittent variations in temperature between 37° C. and 2° C., as compared with a constant thermostat and refrigerator temperature.

Two heavy suspensions in salt solution of diplococci (596 and 598) were kept in the refrigerator, at a temperature of about 2° C., for five days, at the expiration of which time vigorous and abundant growths were still obtained on transplantation. This result has an interesting bearing on the influence of concentration of the suspension of the cocci on their disintegration at higher temperatures.

Three tubes containing vigorous twenty-hour-old cultures of diplococcus (656) were treated as follows: (*a*) was kept constantly at 37° C., (*b*) was alternated daily between the thermostat and refrigerator (2° C.), and (*c*) was kept constantly in the refrigerator. Experiment began November 8. First transplantation November 10. (*a*) and (*b*) abundant growths; (*c*) moderate growth. Second transplantation November 12. (*a*) and (*b*) good growths; (*c*) small number of separate colonies. Third transplantation November 13. (*a*) and (*b*) good growths; (*c*) no growth.

From these tests certain conclusions can be drawn. In the course of growth of the diplococcus, the medium (if solid) is not exhausted of all nutritive value for the coccus in twenty-four to forty-eight hours; and the products of disintegration of the diplococcus are inhibitive to further multiplication of the coccus only when they have accumulated in large quantities. It would indeed appear as if such an accumulation of growth products as to prevent further development could probably not occur in the course of ordinary vegetative existence of the organism; and it is safe to infer that the short vitality of the coccus in cultures is not due directly to the injurious action of these substances. The tests fur-

ther bring out the fact that a fresh and vigorous culture upon a slanting solid surface succumbs quickly when kept constantly at a temperature just above zero, and is less injured by the wide variation of temperatures between 2° C. and 37° C. It could, therefore, be shown that cold is inherently injurious to the cocci, and the injury is inflicted not at once that the low temperature is reached, or equally upon the cocci exposed to it. This fact has an important bearing upon the enzymotic activities of the diplococcus to be described presently. In view of the ease and certainty with which solid cultures are destroyed by cold, the greater resistance displayed by highly concentrated suspensions of the cocci in salt solution is noteworthy. Salt solution, even when changed with washings from the surface of a solid medium, probably does not permit any multiplication of the diplococci; besides which any increase is excluded, in this experiment, by the low temperature. That so many of the cocci survived in the strong suspensions may be due merely to greater vitality of a small number of the cocci, or it may, on the other hand, be due directly to the concentration of the fluid which conceivably exercises a protective influence. This factor of concentration of suspension of cocci is very important in respect to the preservation of the cocci under the conditions of higher temperature when enzymotic action may be presumed to come actively into play, and hence it was desirable to determine whether it played a really important part at the lower temperatures.

A milky suspension of a culture "Smith," twenty hours old, on sheep-serum agar was made in salt solution. The suspension was divided in three parts and used for an experiment at 37° C., 2° C. and —5° C.

The first experiment to be related was made at 2° C. Three tubes were charged with the suspension: (1) was the original strength; (2) contained 0.5 c.c. of the original suspension and 1.0 c.c. salt solution, and (3) contained 0.1 c.c. of the original suspension and 1.0 c.c. salt solution. The first observation was made at the end of twenty-four hours. Cover-glass preparations showed the cocci to be well-preserved in (1), somewhat less sharply-staining in (2) and (3). At the expiration of the second twenty-four hour period, at which time cultures were made, cover-glass preparations showed in all the tubes a moderate reduction in sharpness of outlines and staining of the cocci. The results of the cultivation are important. (1) showed a solid growth of diplococci covering the midportion of the tube; (2) gave sixteen characteristic colonies; (3) gave no growth whatever. Approximately the same volume of suspension, but not the same number of diplococci, was transplanted in each tube.

The deduction from the previous test and this experiment is obvious: cold is injurious to the cocci and destroys cocci in a weak more quickly than in a more highly concentrated suspension. It will not be profitable to discuss at this juncture whether the survival in the concentrated suspension is due directly to its numerical superiority in cocci or indirectly to the existence of more resistant cocci which, as the whole number is more numerous, are also more numerously represented. In even the weakest suspension (3), the cocci transplanted must have been innumerable.

Still further depression of the temperature appears to exert even a greater injury to the cocci. Suspensions of the cocci which were placed, at the same time, at —5° C. failed entirely to grow when transplanted, forty-eight hours later, to a favorable medium, although the morphological changes in the cocci were relatively slight.

It was stated that salt solution is not a medium of growth and multiplication of the cocci. A series of tests was made with salt solution and other media in order to determine the fate of the diplococci introduced into them. The details of the experiment need not, perhaps, be given. A strain of the diplococcus, which by reason of long cultivation outside the body was able to grow feebly on plain agar, was employed. Salt solution (0.85 per cent.), distilled water, and sheep-serum water were inoculated with small quantities of the culture and examined twenty hours later. The salt solution and distilled water showed diplococci in a good state of preservation; the sheep-serum water showed no diplococci. At the expiration of the next twenty-four hours, those in the distilled water were in part fragmented and decolorized, those in the other media remaining unchanged. Growths could not be recovered from any of the fluids.

The observation of the persistence of the diplococci in a dead state in salt solution and water for about forty-eight hours without suffering dissolution is interesting when compared with what takes place, under similar conditions, when the suspensions are heavier. I had observed, in the course of experiments on the virulence of the diplococcus that a concentrated salt suspension tended to go off in virulence for guinea-pigs in a few hours, and, as compared with

the state of preservation of solid cultures of the same age, they were more advanced in involution. This observation led me to a study of the influence of concentrations of salt suspension upon the vitality and destruction of the diplococcus, which gave some interesting results.

If a fresh culture, on a favorable medium, is suspended in salt solution to a milky mixture, and the latter is kept at 37° C., the greater number of the cocci introduced will be so degenerated at the end of twelve to fourteen hours as to be almost unrecognizable. As the suspension is diluted progressively, the degree of degeneration and disintegration diminishes. The relative number of diplococci which survive in these suspensions can be determined by cultures at any period. A single experiment will be given here.

A milky suspension of culture (610) was made in salt solution. Microscopical examination showed innumerable diplococci of characteristic appearance. A series of test tubes were charged as follows and placed in 37° C.

(1) Original suspension.	Micro. exam. after 24 hours; disintegration, shadows, fragments.	After 48 hours: no cocca forms preserved.
(2) Original suspension 1.0 c.c.; salt sol. 1.0 c.c.	Micro. exam. after 24 hours: disintegration slightly less than (1).	After 48 hours: some shadowy coccal forms.
(3) Original suspension 0.5 c.c.; salt sol. 1.0 c.c.	Micro. exam. after 24 hours: disintegration less than (2).	After 48 hours: shadows and deep staining cocci remain.
(4) Original suspension 0.1 c.c.; salt sol. 1.0 c.c.	Micro. exam. after 24 hours: many shadows, no fragmentation.	After 48 hours: best preservation of series.

Cultures made on sheep-serum agar from the supensions kept at 37° C. for twenty hours gave: Original suspension = no growth; dilution (2) = twelve separate diplococcus colonies; dilution (3) = large number of separate and confluent colonies; dilution (4) = two hundred separate colonies.

If this experiment, which was carried out at the thermostat temperature, be compared with the one similar to it in all respects, except that the suspensions were kept two degrees above the freezing point, it will be seen at once that, however the salt solutions may affect the viability of the diplococcus, the influence of concentration on the viability, is diametrically different under the two sets of conditions given. In other words, at the lower temperature more diplococci survive in the concentrated than in the weaker suspen-

sions, while at the higher temperature, more of them survive in the weaker than in the higher concentration. A glance at the microscopical preparation gives a clew to this difference of behavior: while at the refrigerator temperature the diplococci are not markedly disintegrated, at the thermostat temperature degree of disintegration and concentration go hand in hand. Hence, while the diplococci may lose capacity to grow without losing, at the same time, their form and staining reactions, it is probable that once these latter are lost, viability is also lost. Cold, like salt solution, is inherently hurtful to *Diplococcus intracellularis;* but, besides the injury which the salt solution alone inflicts, another detrimental factor comes into action at the thermostat temperature.

Before considering this factor, the question of the manner in which the salt solution acts may be taken up. It must be borne in mind that as compared with the salt strength of the medium on which the diplococcus is cultivated, the 0.85 per cent. salt solution is hypertonic. A. Fischer[5] has shown that passage from weak into more concentrated salt solution produced in bacteria sometimes plasmolysis, sometimes plasmoptysis, and sometimes neither of these changes. Does, therefore, the change of medium in this case bring out plasmoptytic alterations of the diplococci? Three facts speak against the changes described as being plasmoptytic. The first is the influence of concentration of the bacterial emulsion, the second is the effect of low as compared with higher temperatures, and the third, now to be described, is the influence of a salt solution of 0.9 per cent. concentration containing calcium and potassium chloride. If suspensions of the diplococcus are made in Ringer's solution the viability of the coccus is greatly prolonged as compared with the survival in the salt solution, or, what is even more remarkable, upon the sheep-serum agar. A series of Ringer's solution's suspensions of the diplococcus of three concentrations: (1) Milky suspension, (2) first suspension 0.5 c.c. and Ringer's solution 1.0 c.c., (3) first suspension 0.1 c.c. and Ringer's solution 1.0 c.c., kept at 37° C. survive many days. I have observed that on the twelfth day a considerable number of the diplococci in the original milky suspension was still viable, a smaller number was still viable

[5] *Zeitschr. f. Hygiene u. Infectionskrank.*, 1900, xxxv, 1.

in the suspension of medium concentration, while no growth was obtained after the fifth day from the lowest concentration. All the diplococci had died out in the original suspension on the fifteenth day. This experiment establishes several important points: it proves that the death of the diplococcus is not caused by hypertonicity of medium alone, by starvation alone, by degree of concentration alone, or by a naturally brief period of vitality. If frequent microscopical examinations of the suspensions in Ringer's fluid are made a gradual dissolution of the diplococci can be made out, but the disintegration progresses slowly, as compared with the effect of salt solution, and the sharp distinction between the different concentrations is lost. I have noticed in those suspensions which gave subcultures a change in appearance of a number of the diplococci which may be connected with the power of survival. These individuals, which are somewhat larger than the average, and take on a deep safranin stain, displaying a high degree of refraction, exhibit a greater resistance to the disintegrative influences. I had already observed these morphological forms in my experiments upon the destructive action of exudates upon the diplococcus, and the question occurred to me whether they represent a more enduring type of the micro-organism. I am unable, at present, to answer this question.

A simple experiment has shown that the addition of a salt of calcium to an otherwise suitable culture medium will greatly increase the period of viability of the diplococcus which has grown upon it. If calcium carbonate is added to the sheep-serum water agar, a given culture will survive many weeks. A rough experiment showed that at the end of five weeks a strain which otherwise survives, on the medium minus the calcium salt, only two, three or four days, was still alive and able to yield abundant sub-cultures. While at the end of five or six days, the original cultures show no preserved, but only disintegrated diplococci; the growth on this new medium two weeks old still showed innumerable diplococci of faint staining power.

The experiment at 37° C. with the Ringer's solution indicated that the survival of diplococci was not necessarily in inverse ratio to the concentration of the suspension, but was in ratio to the con-

centration. In this respect the influence of Ringer's fluid is widely different from that of salt solution, and agrees with the effect of cold on the survival of the diplococcus in salt suspensions. It was deemed desirable to ascertain whether cold, as such, exercised as injurious an influence upon the diplococcus suspended in Ringer's solution as it was shown to exercise on agar cultures and on salt suspensions. Diplococci suspended in Ringer's fluid and kept at 2° C. survived eight to ten days, which is about three times as long as they survived under the other conditions. This period is behind that of survival at 37° C., whence it follows that cold as such is more injurious than warmth. Diplococci could be subcultured longer from the stronger than from the weaker suspensions kept at 2° C.; and as the power to grow in subcultures was lost disintegration of the diplococci was observed to take place extensively. Exceptions to this rule may be observed. A highly concentrated suspension in Ringer's fluid of a vigorous looking diplococcus culture failed, in one instance, to survive 24 hours at 2° C.

It must, therefore, be concluded that salt solution is directly injurious to *Diplococcus intracellularis,* as it has been shown by Jacques Loeb[6] and others to be poisonous to many animal cells; and it must be belived that the toxic effects of sodium chloride can be neutralized by certain calcium and potassium salts. Although the salt solution causes directly the death of the diplococci, it would appear that it is not to it, but to some other agency, to which must be ascribed the disintegration which rapidly overtaxes the dead micro-organisms.

The nature of this agency is rendered fairly evident from the microscopical appearances presented by the suspensions: it would seem to be enzymotic. The manner of dissolution of the diplococci suggests this, and the enzyme is doubtless contained in the bacterial cells. As enzymes act with energy not upon highly vitalized, but upon dead or partly devitalized cells, the question arises whether in the case of the diplococcus the enzyme can act immediately as a devitalizing as it can as a dissolving agency. It has been shown that in weak salt suspensions the diplococci survive and retain their morphological integrity longer than in stronger suspensions; and

[6] *Biochemische Zeitschrift,* 1906, ii, 81.

hence, it may be conceived that while the charges of enzyme which individual diplococci carry may be inadequate both to destroy and to disintegrate them, that in the more concentrated suspensions the enzyme liberated from a larger number of disintegrating microorganisms, may exert a devitalizing effect. The intracellular enzyme is, therefore, a dissolvent of dead diplococci, and in certain states of concentration a poison for living ones.

The action of the enzyme upon the bacterial cells can be accelerated by the employment of chemical and physical agents which serve to kill the diplococci without, at the same time, seriously injuring the enzyme. Heat, carefully applied, and toluol have been used to accomplish this purpose. Suspensions of the diplococcus heated for thirty minutes to the temperature of 60° C. are killed and still capable of undergoing disintegration or autolysis. The ultimate result is the same whether the suspension has been made in salt or Ringer's solution; while in point of rapidity the dissolution is somewhat slower than in strong unheated suspensions in salt solution. Heating to 65° C. and higher temperatures, reduces the capacity of the diplococcus to undergo rapid disintegration, probably because the enzyme is weakened or destroyed.

If to a strong salt suspension of the diplococcus toluol be added and the mixture kept at 37° C., the morphological changes indicative of autolysis begin to be evident in one hour, to be pronounced in two hours, and nearly complete in four hours. Ringer's fluid has no marked influence on this form of autolysis. It is interesting to note that the degree of concentration of the suspension influences the result in the presence as in the absence of toluol. Even the most concentrated salt suspensions alone do not show appreciable changes in the morphology of the diplococcus at the expiration of four hours at 37° C. The tabulation which follows brings out the main facts.

If Series I and II in the succeeding tabulation are closely scrutinized, such differences in degree of disintegration can be made out as to indicate that toluol not only accelerates autolysis of the cocci, but it tends also to make it more complete and to minimize, but not to set aside entirely, the effect of the degree of concentration upon the final disintegration.

A milky suspension of diplococcus (656), showing under the mircroscope innumerable diplococci, was prepared. Series I consisted of salt suspensions of cocci alone, Series II of salt suspensions to which toluol was added. The examination was made after eighteen hours at 37° C.

SERIES I.

Original suspension.	Extensive disintegration; small number of coccal forms still visible.
Original suspension, 1.0 c.c.; salt sol. 1.0 c.c.	Less disintegration than previous one.
Original suspension, 0.5 c.c.; salt sol. 1.0 c.c.	Less disintegration than previous one.
Original suspension, 0.2 c.c.; salt sol. 1.0 c.c.	Fair degree of preservation of cocci.
Original suspension, 0.1 c.c.; salt sol. 1.0 c.c.	About same degree of preservation as previous one.

SERIES II.

Original suspension + toluol.	Complete disintegration.
Original suspension 1.0 c.c.; salt sol. 1.0 c.c. + toluol.	Complete disintegration.
Original suspension 0.5 c.c.; salt sol. 1.0 c.c. + toluol.	Disintegration incomplete; pale and irregular coccal forms visible.
Original suspension 0.2 c.c.; salt sol. 1.0 c.c. + toluol.	Somewhat better preservation than previous one.
Original suspension 0.1 c.c.; salt sol. 1.0 c.c. + toluol.	Better preservation than previous one.

Jacques Loeb[7] has drawn attention on several occasions to the restraining influence of potassium cyanide upon intracellular enzymotic activity. The diplococcus offered, therefore, the opportunity to study the effects of this chemical body upon an active autolytic ferment under the conditions of purity of culture of microorganic cells—conditions probably not obtainable with the cells of higher living forms.

The experiments were made with different strengths of the cyanide and suspensions of the diplococci. Potassium cyanide in 1/200 per cent. solution kills the diplococci outright. If now the diplococci, in watery suspensions, are left in contact with the cyanide, either with or without the addition of toluol, the organisms do not disintegrate, and suffer only reduction in their affinity for

[7] *Biochemische Zeitschrift,* 1906, i, 183.

dyes. That enzyme activity is not wholly suppressed by the cyanide would seem to be indicated by the better state of preservation of the cocci in the dilute suspensions. The tabulation (Series III) brings out this difference. The suspensions and examination of the diplococcus were made at the same time as those of Series I and II of the preceding tabulations.

SERIES III.

KCN 1/20% 1.0 c.c., susp. cocci 1.0 c.c.	Outlines of cocci pale but visible; possibly reduction in numbers.
KCN 1/20% 1.0 c.c., susp. cocci 0.5 c.c.	Fair preservation of the cocci; no reduction in numbers.
KCN 1/20% 1.0 c.c., susp. cocci 0.2 c.c.	Good preservation of the cocci.
KCN 1/20% 1.0 c.c., susp. cocci 0.1 c.c.	Very good preservation; outline of cocci not perfectly distinct.
KCN 1/20% 1.0 c.c., susp. cocci 0.01 c.c.	The same as previous one.

This experiment shows that potassium cyanide possesses the power of preventing or diminishing autolysis of the dead diplococci. In the case of the higher concentrations it is even possible that a greater quantity of the cyanide may have wholly suppressed autolysis. Doubtless many other chemical agents possess this power of suppressing autolysis of the diplococcus; and hence it was necessary to ascertain whether the removal of the cyanide will admit the enzyme to reassert its enzymotic action, as, according to Loeb, happens in the case of the eggs of the sea-urchin. If, therefore, the cyanide acts by holding the enzyme in check and not by destroying it, or changing the cell-substance, so as to make it permanently resistant to the action of the enzyme, the diplococci should be restored approximately to their normal condition of autolysis upon the removal of the cyanide.

Ten cubic centimeters of a strong suspension of the diplococcus in salt solution were mixed with one cubic centimeter of a one twentieth per cent. potassium cyanide solution, and the mixture kept at 37° C. for one hour. The cocci and fluid were separated by centrifugalization and the coccal residue washed twice with 10 c.c. of salt solution. The sediment now showed a large number of well-preserved, deeply-staining diplococci. The sediment was resuspended in salt solution and divided into two portions, to one of which toluol was added. They were placed at 37° C. and examined at the end of twenty-four hours and forty-eight hours. No marked difference was noticed in the toluol and non-

toluolized specimen. The diplococci appeared reduced in number, evident disintegration was in progress, and the sharpness of staining had been lost at the first examination. At the second examination, the staining was still more feeble, but no other change was noticeable.

From the experiments, it can be stated that the diplococci, after treatment with the cyanide can be partially restored to their previous state of autolysis. The experiment is not conclusive of the actual degree of restoration possible, since the disintegration of the cocci was incomplete in the experiment. The concentration of the resuspension, which was not high, may explain the imperfect disintegration. It would, indeed, be possible to conduct this experiment quantitatively and ascertain the degree of permanent injury exercised by the cyanide upon the diplococcus; but the object of the experiment in establishing the restraining action of potassium cyanide upon the autolytic enzyme, and the possibility of setting this action aside by mechanical removal of the poison, was attained by the imperfect experiment.

The existence of an autolytic enzyme in *Diplococcus intracellularis* capable of destroying its own cell-substance having now been established, the next step was to ascertain whether the enzymotic action was specific and limited to the diplococcus, or whether it could be exerted upon other bacterial cells. It is known that the diplococcus does not produce a proteolytic enzyme acting upon and dissolving gelatine and coagulated serum. Hence, its enzyme differs materially from the enzymes secreted by certain liquefying bacteria. It seems quite certain that the rapid and striking morphological changes taking place in cultures of the diplococcus are caused, directly or indirectly, by the intracellular enzyme; and it may, therefore, in view of this fact and the related ones already described, be presumed that the diplococcal enzyme is able to break down the complex structures contained in living micro-organisms.

The enzymotic action of the diplococcus was tested upon the two classes of bacteria represented by those staining and those decolorizing by Gram's method. Among the latter, *Bacillus typhosus, Bacillus coli communis, Bacillus pyocyaneus, Micrococcus catarrhalis* and two unidentified Gram negative cocci from the monkey's nose were tested; while among the former *Staphylococcus aureus* and *Bacillus anthracis* were chosen.

The experiments to determine this point were made in the following manner: Fresh growths upon agar-agar surfaces were suspended in salt solution to a milky emulsion which was divided into two equal parts. To one part an equal volume of salt solution, and to the other the same volume of a milky suspension of *Diplococcus intracellularis* was added. Control cover-glass preparations were made immediately and double-stained with gentian-violet and safranin (Gram's method).

Toluol was now added, and the tubes placed at 37° C. under rubber caps. The examinations were made by means of cover glass preparations (Gram's method), usually at twenty-four hour intervals. A few detailed results follow.

Non-Gram coccus from monkey. (*a*) Controls: Original suspension shows many sharply-staining cocci, single and in pairs; mixed suspensions of coccus and diplococcus permit easy distinction by the morphological differences. After twenty-four hours: Original suspension shows no solution or disintegration of the cocci; outlines of the cocci not quite sharp. Mixed suspension: All the diplococci have disintegrated; the larger cocci from the monkey have lost stainable substance and have run together into small masses in which the individual outlines are indistinct. After forty-eight hours: Original suspension, cocci somewhat paler and cohering slightly. Mixed suspension: Reduction in number of cocci; those remaining appear as shadows. The non-Gram coccus (*b*) gave results which were almost identical with those of (*a*).

B. typhosus and *B. coli communis* in milky suspensions under toluol, are somewhat altered in appearance at the end of forty-eight hours at 37° C.; while in the presence of an emulsion of the diplococcus, such as was used with the non-Gram cocci from the monkey, they are completely or almost completely destroyed in this period. At the expiration of the first twenty-four hours, the disintegration is advanced.

Bacillus pyocyaneus.—The control under toluol showed, at the end of twenty-four hours, no reduction in number, but less deep staining than the preparation made immediately after suspension. The suspension with the diplococcus showed, at this time, reduced numbers, and only shadowy remains of bacilli. At the expiration of the second twenty-four hour period, the control tube still showed many stainable bacilli (number apparently less than in original suspension), while the mixed tube containing the two organisms was completely disintegrated.

Two cultures of *Micrococcus catarrhalis,* which were found to be quite resistant under toluol, were found to disintegrate quickly (in twenty-four hours) under the influence of the suspension of the diplococcus.

Staphylococcus aureus.—Control (under toluol) showed no appreciable change after several days at 37° C. The mixed suspension of staphylococcus and diplococcus was examined at twenty-four hour periods for five days. After twenty-four hours, there is reduction in numbers of the staphylococci, although many

still remain. In size these are larger than in the control tube, and the violet staining is less intense. In the succeeding days, the number of cocci further diminished, the cocci coalesced into clumps, and showed still further reduction in power to hold the violet stain. Here and there throughout the preparation, transitions from staphylococci holding the violet stain imperfectly to staphylococci which had taken up the counter-stain (safranin) were observed. At the expiration of the experiment, not all the staphylococci had disappeared or had been decolorized.

Anthrax bacillus twenty hours old on slant agar; no spores visible. Suspended as before: First observation after twenty-four hours. Control (under toluol): Partial and moderate degree of plasmolysis. The threads and felted masses of bacilli show clearly an outer-stained (safranin) cell-wall and an inner-stained (violet) cell-substance. The inner substance is somewhat fragmented and irregularly contoured. Mixed suspension: Advanced plasmolysis and loss, in high degree, of Gram-staining inner substance. The outer cell-membrane is completely gone in many places, and the fragmented or partially dissolved inner substance lies free. Second observation, after forty-eight hours: Control unchanged. Mixed suspension: Practically complete decolorization of the threads, with very few fragmented remains of violet-staining inner cell-substance.[8]

It is established by the foregoing series of tests that the enzymotic action of the diplococcus can be exercised on a considerable variety of bacteria, and the observations are not without interest as indicating that micro-organisms, such as *Staphylococcus aureus, Bacillus anthracis,* and *Bacillus pyocyaneus,* in spite of their energetic proteolytic functions, show less power to cause disintegration of their own protoplasm than is effected by *Diplococcus intracellularis.* This difference points to a distinction between autolytic and proteolytic bacterial enzymes. The above tests also showed that a great difference exists in regard to the ease and completeness with which this hetero-disintegration of bacterial cells is produced by the diplococcus. Of the bacteria studied which were at all subject to the enzyme *Staphylococcus aureus* offered the greatest resistance; and I think it not improbable that the Gram-positive bacteria in general may prove to be less readily broken down by the diplococcus enzyme than the Gram negative bacteria.[8] Eventually, the bacteria which produce proteolytic enzymes, and, indeed other bacteria, suffer more or less complete disintegration. In the case of *Bacillus*

[8] The destruction of *Staphylococcus aureus* was incomplete, the change in *Bacillus anthracis* was one of degree of disintegration; the changes in the other bacteria were more striking. No effect was noticed in tests with *Bacillus tuberculosis* and *Bacillus Moelleri* (timothy bacillus).

pyocyaneus, in salt suspension in toluol, the time required for disintegration is, at most, a few days. But the slower forms of involution and degeneration observed in artificial cultures, may or may not be of the nature of the autolytic changes described. These involutional alterations are probably of extremely subtile nature; but even here enzyme activities of quite low grade may be the chief agencies at work.

The heat lability of the enzyme has been studied in two ways. First, the temperature at which the diplococcus is prevented from undergoing characteristic autolysis was determined. Next, the

The suspended contents in sheep-serum water of surface growths from two-pint Blake bottles were distributed among ten test tubes; each tube received about 6 c.c. The tubes were, with the exception of the controls, heated for thirty minutes to temperatures ranging from 55° C. to 90° C. Cultures were made to determine whether any diplococci remained viable. Only the control (unheated) tubes gave growth. Cover-glass preparations were made from the control tubes, and from the heated tubes immediately after heating and at the subsequent periods. The tabulation gives the results:

Suspension.	Immed. Microscop. Appearance.	Microscop. Appearance 24 Hours Later.	Sedimentation After 3 Days.
Control.	Well-preserved cocci.	Marked degeneration; diminution of cocci.	Cloudy; no sediment.
Toluol control	Well-preserved cocci.	A few minute, pale fragments remain.	Almost clear; no sediment.
55°C.	Cocci stain less sharply.	Some cocci preserved; many shadows and fragments.	Almost clear; mod. sediment.
60°C.	Cocci stain less sharply.	Some cocci preserved; many shadows and fragments.	Almost clear; mod. sediment.
65°C.	Some frag. and decol. cocci.	Somewhat more fragmentation than at 55°C.	Almost transparent; much sediment.
70°C.	Some frag. and decol. cocci.	Forms better preserved than at 65°C.	Almost transparent; much sediment.
75°C.	Some decolorization.	Preservation very good.	Almost transparent; much sediment.
80°C.	Considerable fragmentation.	Preservation good, but much irregularity in staining.	Almost transparent; much sediment.
85°C.	Frag. and apparent diminution in number.	Staining deep; no shadows; irregular forms.	Almost transparent; much sediment.
90°C.	Frag., diminution in number; decolorization.	Forms pale; no further diminution.	Almost transparent; much sediment.

temperature required to render the autolysate of the diplococcus inactive upon other bacterial species was ascertained.

A temperature of 55° C., maintained for thirty minutes, kills the diplococcus, but it does not suffice to remove its capacity for self-digestion. Even after exposure, for the same period, to a temperature of 60° C., the diplococcus undergoes autolysis. This test was several times repeated with different strains of the diplococcus and autolysis was always observed to occur, although somewhat less quickly and perfectly than in unheated coccal suspensions under tuluol. Toluol accelerates, I believe, dissolution, by causing plasmolysis and thus promoting the disintegrative effect of the enzyme. The thermal death points of cocci and enzyme are widely separated. Temperatures of 40° C. to 45° C. are capable, if maintained some hours, of killing the diplococci, while the enzyme does not suffer injury under the same conditions. Temperatures of 65° C. and upwards, while causing more or less immediate plasmolysis (or plasmoptysis) prevent further disintegration due to a vigorous action of the enzyme. As the tabulation indicates, two criteria can be used to determine the point of heat-destruction of the enzyme: first, the degree of dissolution shown by the microscope, and next the amount of sediment appearing in the tubes. The two effects stand in a quantitative inverse ratio to each other. The greater the amount of sediment, the smaller the amount of disintegration of the diplococcus as shown by the microscope. Hence the gross test is well adapted to the study of the lability of the enzyme, since it permits of the elimination of any error arising from mere physical or mechanical change in the diplococci.

To ascertain whether the temperature at which self-digestion of the diplococcus is inhibited represents the temperature of heat-destruction of the enzyme, another series of tests was made.

The point of heat injury or destruction of the enzymotic fluid was determined by preparing, in pint Blake bottles, mass cultures of the diplococcus and causing them to autolyze at 37° C. for forty-eight hours in salt solution and in water under toluol. At the expiration of this period the toluol had evaporated. The salt autolysate was distributed in test tubes, of which some were heated to 70°, 80°, 90° and 100° C. for thirty minutes, and some remained unheated. A coagulum formed in the tubes heated to 90° and 100° C. The watery autolysate was not heated. The contents of all the tubes were centrifugalized; and except

those heated to 90° and 100° C., which became transparent, the fluids remained opalescent. Twenty hours' growths of *B. coli communis, B. typhosus* and *B. anthracis* were suspended in Ringer's fluid to milky emulsions. The tests were made as follows: A set of test tubes was charged with the unheated salt and water autolysates and the salt autolysate heated as described. To the control tube an equal amount of salt solution was added. An approximately equal amount of the milky bacterial emulsion was now placed into each tube, toluol was added, and the mixtures bept at 37° C. for forty-eight hours. Two examinations at tweny-four-hour periods were made. The result in the case of *B. coli communis* was definite: the control salt suspension showed the bacilli swollen, pale and shadowy, but not disintegrated; the salt and water unheated autolysates showed complete disintegration of the bacilli; the autolysate heated to 70° C. was scarcely to be distinguished in effect from the unheated; while the autolysates heated to 80° C. and higher produced about the same degree of change as the salt control. The result in the case of *Bacillus typhosus* was disappointing, as, in this experiment, the salt control as well as the autolysate mixtures disintegrated completely. A perceptible difference was manifest in the degree in which *Bacillus anthracis* disintegrates in toluolized salt solution and in a mixture of equal parts of salt and Ringer's solution. The addition of the latter reduced the spontaneous disintegration. In like manner the unheated autolysates are less active destructive agents where Ringer's fluid is present, as compared with their action in plain salt solution. And yet the disintegration was greater when the unheated autolysate was present. The autolysate heated to 70° C. and 80° C. produced less change, and that heated to 90° C. and 100° C. still less, than took place in the unheated salt and Ringer's fluid control.

While this experiment is not perfectly sharply cut, it yet indicates that heating the fluid autolysate to 70° C. and above reduces its powers of causing disintegration of certain bacteria.

PATHOGENICITY OF THE DIPLOCOCCUS.

Diplococcus intracellurais is, as tested on laboratory animals, a micro-organism of low and variable pathogenic action. I have studied its action upon mice, guinea-pigs, rabbits and monkeys. Different strains of the diplococcus were injected into different regions of the body to determine the fatal doses and the pathological effects produced by the micro-organism. In the case of the monkey the inoculations were made directly into the spinal canal, in order to reproduce the symptoms and lesions of cerebro-spinal meningitis in man. The experiments to be related immediately refer to the action of the diplococcus upon the animals mentioned, except the monkeys. The experiments on monkeys form the subject of a separate communication.

A considerable number of strains of *Diplococcus intracellularis* have been tested for virulence upon mice and guinea-pigs. In all the later experiments upon the virulence of the micro-organisms I preferred to employ small guinea-pigs, as being more susceptible to the action of the diplococcus. The difference in resistance to infection in guinea-pigs, depending upon weight, is considerable. Guinea-pigs of 175 to 200 grams weight have proved highly susceptible to the diplococcus, compared to the resistance displayed by pigs weighing 350 to 400 grams. Mice of about 15 grams weight often withstand larger doses of the diplococcus than the smaller pigs.

Not all cultures of the diplococcus, even in the first generations, are even moderately pathogenic for guinea-pigs and mice, although it is always possible to kill these animals by inoculating an excessively large quantity of cultures of low virulence. Speaking generally, the freshly isolated cultures are much more virulent than cultures grown for a period on artificial media. Occasionally, a strain of the diplococcus will retain its virulence for many months, but, as a rule, this power becomes greatly diminished, or is lost, after the first transplantations extending over a few weeks. I have worked with many cultures which deteriorated in a few days, and one culture (Smith) that retained its virulence for many months, to lose it in the end, and another (656) that displayed no diminution in activity after several months cultivation on sheep-serum agar. The "Smith" culture was passed through many mice, guinea-pigs and monkeys during its active period; but once its power was lost, it could not be restored to virulence by passage through mice or guinea-pigs. It still yields, however, by autolysis, an active extract and can be used as an adjuvant to increase activity of other living cultures.

In any comparison of virulence little weight can, I think, be laid upon the absolute quantities of diplococci injected. The animals are, on the whole, so refractory that the number of diplococci that must be injected to produce striking results is, even with the most active cultures, very large. Very little accuracy could in my work be secured through the use of a standard *oese,* for the reason that the contained water in the surface growth was subject to wide

fluctuations, depending upon the strain of diplococcus, composition of the culture-medium, and other external factors.[9] Hence, I substituted for the *oese* a suspension of the diplococci, incubated for a uniform period (usually 20 hours) upon a standard medium (7 c.c. glucose-agar to 1 c.c. sheep-serum water). The suspensions exhibited a degree of opacity which was approximately equal. Select-

No. of Pig.	Weight in Grams.	Denomination and Quantity of Culture.	Result.
113	165	0.1 c.c. No. 654.	Survived.
119	160	0.2 c.c. "	"
121	177	0.5 c.c. "	Died 9 P.M. same evening.
122	192	0.1 c.c. No. 88.	Survived.
127	175	0.2 c.c. "	Died.
128	182	0.5 c.c. "	"
145	197	0.1 c.c. Buck.	Survived.
150	195	0.2 c.c. "	"
159	170	0.5 c.c. "	Died.
161	165	0.1 c.c. No. 9.	Survived.
162	175	0.2 c.c. "	"
163	166	0.5 c.c. "	"
164	161	0.1 c.c. Connolly.	Survived.
166	190	0.2 c.c. "	"
172	195	0.5 c.c. "	Died.

[9] An attempt was made with four cultures of the diplococcus to increase the virulence by growing the diplococci in a glucose-bouillon medium containing one twentieth of its volume of immune goat serum. For the controls normal goat serum was employed. Each culture was passed at twenty-four-hour intervals through ten successive tubes of medium. The tenth cultures were injected into the peritoneal cavity of mice. As the tabulation indicates, no augmentation was accomplished. The immune serum caused agglutination of the growing diplococci. The mice dying in twenty-four hours showed, as is usual, general infection with the diplococcus. Mr. Herbert Ward assisted me in these experiments.

Quantity of Culture Injected.		Immune Series Result.	Normal Series Result.
Kepp culture.	3 c.c.	Died in 15 hours.	Died in 10 days.
	2 c.c.	Survived.	Survived.
	1 c.c.	"	Died in 10 days.
	0.5 c.c.	"	Survived.
	0.1 c.c.	Died in 5 days.	Died in 10 days.
Behren culture.	3 c.c.	Died in 15 hours.	Died in 24 hours.
	2 c.c.	Survived.	Died in 20 hours.
	1 c.c.	Died in 24 hours.	Survived.
	0.5 c.c.	Survived.	"

ing a lot of guinea-pigs of about equal weight approximately uniform quantities of the cultures could be injected.

The preceding tabulation will suffice to show the method of testing comparative virulence and give the results obtained in a typical experiment.

One agar-slant culture of each strain was suspended in about 3 c.c. of salt solution. Injections made intraperitoneally at 5 P. M. The pigs succumbed during the night or the next morning.

As salt solution is evidently an active poison for the diplococcus, and as sheep-serum water favors, when added to an otherwise suitable medium, the growth and preservation of the diplococcus, an experiment was made to ascertain whether suspending the diplococci in one or the other fluid influenced the pathogenic result. The experiment was made with a culture on sheep-serum agar eighteen hours old. The suspensions were prepared at 11 A. M., and the injections given intraperitoneally at 4 P. M. In point of concentration the salt suspension was, as indicated by the microscopical field, considerably the heavier.

No. of Pig.	Weight in Grams.	Quantity of Suspension Injected.	Result.
102	230	1.0 c.c. salt.	Died on second day.
121	267	0.5 c.c. "	Survived.
134	260	1.0 c.c. serum.	Died on first day.
130	270	0.5 c.c. "	Died on second day.
190	115	0.2 c.c. "	Died on first day.

This result merely suggests that as compared with a sheep-serum water suspension the salt suspension of diplococci suffers a more rapid deterioration. In certain experiments on immunity which I carried out I observed that it was unsafe to carry the salt suspensions of the diplococci over, from one day to the next, without making a new determination of the strength. The apparently better preservative action of serum-water over salt solution is the more remarkable in that the serum water is not a favorable medium of growth for the diplococcus. Indeed, many strains are unable to develop in it alone; and the transplanted diplococci, which do not multiply, soon disappear. If equally heavy suspensions, say an agar-slant surface growth per 4 c.c. of fluid, of the diplococcus are made in salt solution and sheep-serum water and kept at 37° C.,

the disintegration is more advanced at the end of twenty-four hours in the salt than in the serum-water.[10]

In studying the pathogenic action of the diplococcus salt suspensions were invariably used. Where a large number of animals was to be inoculated, the diplococcus was grown on the flat agar-covered surface of a one-pint Blake bottle. A suspension of the growth in 12 c.c. of fluid was made of which the fatal dose varied, according to the virulence of the diplococcus, from 0.1 to 0.5 cubic centimeter for small guinea-pigs, by intra-peritoneal inoculation. Death from these doses occurred, as a rule, in less than twenty-four hours. Active cultures, in this strength of suspension, may in the smallest dose given cause death in from eight to ten hours. A culture of which a dose of 0.5 c.c. requires twenty-four hours to cause death is one of low virulence. In the course of any large series of experiments, irregularities in the reaction of guinea-pigs to inoculation occur. On the whole, the irregularities occasioned by greater or less resistance on the part of the small pigs are remarkably few. It has, indeed, rarely happened that a guinea-pig of a series died in four hours and another survived thirty-six hours. But these irregularities can, when required, as in tests of immunity, be ruled out by making duplicate inoculations. Care should be exercised not to employ guinea-pigs weighing less than 150 to 160 grams, the most useful weights being about 200 grams. Among the pigs surviving inoculation immediately, it happened, now and then, that death took place after several days, or a few weeks, and was preceded by a state of great emaciation. No constant pathological condition, the emaciation excepted, was found in these animals. The peritoneum was usually clear, and no diplococci remained there or elsewhere in the body.

One of the earliest visible results of the inoculations is a marked reduction in temperature. This is associated, at times, with the characteristic external appearance of sick guinea-pigs: the tendency to avoid the light, and to sit crouched in a corner with the hair erected. The abdominal muscles become tense and hard and the abdomen distended. The irritative effects of the inoculation upon

[10] The directly toxic action of sodium chloride upon the diplococcus has already been described.

the intestine are associated occasionally with prolapsus of the rectum which condition is followed invariably by death. The rapid onset and high degree of reduction of temperature following inoculation permit frequently the prediction in two or three hours of the probable outcome. Hence, it is possible, in many cases, to determine in a short time the result of tests upon virulence of the diplococci, the protective value of immune sera, etc. In testing immune sera it is necessary in each instance to make a preliminary estimation of the activity of the organisms, since the concentration of the suspension and the degree of virulence are such variable factors; and as the suspensions cannot be kept in salt solution for twenty-four hours without changing, and should be injected as soon as possible after preparation, it is an advantage to have this relatively safe guide to further inoculations of the temperature depression. The tabulation will exhibit the effects described.

No. of Pig.	Weight in Grams.	Amounts of Inoc. 12 M.	Temp. Before Inoc.	Temp. and Condition 3 P. M.	Result.
113	170	0.1 c.c. suspension.	38° C.	35.3°C. abdom. tense.	Died 12 M. next day.
119	160	0.2 c.c. suspension.	37.6° C.	34.1°C. " "	Found dead 7 A.M. next day.
121	186	0.3 c.c. suspension.	38.1° C.	35.1°C. " "	Died 9:30 P.M. same day.
122	170	0.5 c.c. suspension.	38.1° C.	34.8°C. " "	Died 9:30 P.M. same day.

As a control for this series the following tabulation will suffice. The temperature of the pigs prior to inoculation was about 38° C. The quantities of emulsion of the several strains of diplococci injected ranged from 0.1 to 0.5 cubic centimeters. The injections

Weight in Grams.	Quantity of Suspension Injected.	Temp. after Inoc.	Result.
165	0.1 c.c.	36° C.	Survived.
160	0.2 c.c.	37.2° C.	"
182	0.5 c.c.	35.6° C.	Died.
{165	0.1 c.c.	35.4° C.	Survived.
{175	0.2 c.c.	38.6° C.	"
{166	0.5 c.c.	38.4° C.	"

were made at 6 P. M., and the temperature of the surviving pigs taken the next morning.

The value of the tabulation immediately preceding consists, first,

in showing that the temperature depression is less in cases in which the inoculation is not fatal, and second, the degree of resistance of the individual pigs is a factor to be reckoned with. The bracketed series illustrates the latter point: the same suspension of diplococcus was injected into the three pigs, and the pig which received the smallest dose showed the greatest depression of temperature. A similar effect has been noted in other cases, but, on the whole, is rather exceptional.

The fate of the diplococci injected into the peritoneal cavity in guinea-pigs depends upon several conditions: the quantity of diplococci injected, the virulence of the diplococci, somewhat upon the individual resistance of the pig, since this is a factor in prolonging or diminishing the period of survival, and the extraneous condition of elapsed time between death and autopsy. As will appear later, the diplococci are not highly resistant to the destructive action of the fluids of the body whether they act upon them in the peritoneal cavity or in the test-tube. Some experiments tend to show that the more virulent diplococci resist the disintegrative action of the fluids in the body longer than the less virulent ones. Comparison of the conditions arising in the peritoneum, depending upon the quantities and the virulence of the diplococci injected, offers a possible explanation of this difference.

The more virulent the diplococcus the shorter is the period of survival after inoculation. Guinea-pigs which succumb in less than eighteen hours—and many succumb to injection of virulent diplococci in ten to fourteen hours—tend to develop a peritoneal exudate poor in leucocytes; while the pigs which survive longer, *i. e.,* from twenty-four to thirty-six hours, tend to exhibit turbid exudates rich in leucocytes. No absolute ratio between the number of leucocytes in the exudate and the degree of disintegration of the diplococci exists; but a relative ratio seems to exist. With an increasing period of survival there is greater emigration of leucocytes and also greater disintegration of diplococci. Hence, the degree of emigration of leucocytes comes to be a measure, in part, of the degree of virulence of the diplococci. The relation can, however, be disturbed either by the inoculation of very large quantities of little virulent, or sublethal doses of the more virulent diplococci, as

might indeed be expected. It is, however, to be pointed out that large overdoses of the weaker diplococci can be readily disintegrated in the peritoneal cavity, even though emigration of leucocytes is largely prevented by the excessive dose. From this fact the result may be deduced that the destruction of the diplococci does not take place necessarily within leucocytes, but can be accomplished by fluid inflammatory exudates alone. As will be seen presently, test-tube experiments bear out this deduction. There is similarity in the pathological conditions met with in cerebro-spinal meningitis in man and in the experimental infection in guinea-pigs. In both species the immediate local reaction to the presence of the diplococcus in the serous cavities is an exudation of fluid, the character and quantity of which varies greatly, perhaps with the quality of the infecting diplococcus. The less cellular fluids often contain the greater number of diplococci of well-preserved appearance which are readily cultivated; while the more cellular fluids often show fewer diplococci and yield cultures with greater difficulty. No striking relation need exist between the virulence of the diplococcus as displayed for man and animals, since the inherent qualities in the germ which determine pathogenicity are often quite dissimilar for different classes of animals.

I have pointed out that some relation between virulence of diplococcus, period of survival of the inoculated guinea-pigs and the emigration of leucocytes exists, and it remains to add that phagocytosis, in the peritoneal cavity, while a variable factor is a fairly constant one. When emigration of leucocytes is at all marked, even with an almost entire preservation of the diplococci, phagocytosis is occurring. When there has been considerable, although not total, disappearance of the free diplococci, phagocytosis is likely to be more abundant. It rarely happens that all the diplococci are within phagocytes unless a small number only of those injected remain. On the other hand, when overdoses of little virulent diplococci are injected they may disappear as stated almost wholly from the peritoneal cavity although the emigration of leucocytes may have been entirely suppressed; or, as is also possible, the emigrated leucocytes may have undergone complete lysis. Hence, I have concluded first, that phagocytosis, in this experimental disease, is a

function of emigration but not a measure of it, and second, the disappearance of the diplococci from the peritoneal cavity does not depend wholly on phagocytosis. I believe, indeed, that the diplococci can be removed, without direct intervention of phagocytes, through self-digestion and digestive action of the inflammatory exudate. The ease with which the diplococcus undergoes autolysis has already been dealt with; and that the inflammatory exudate exerts actively destructive effects upon the diplococcus will be shown presently.

Leucocytes are numerous in every fibrinous exudate, and frequently, in the omentum, when they may be sparse in the peritoneal fluid. Speaking generally, the clear peritoneal fluids contain few leucocytes relatively, and the turbid fluids contain many. The leucocytes of the omentum are large and pale, and those of the peritoneal fluid small and dense. Their nuclei are often irregular in form, or fragmented. Diplococci are common in the protoplasm and rare in the nuclei, and they show all transitions from well-preserved, sharply-staining organisms to fragments of cocci and swollen, metachromatic bodies. Degenerations are exceedingly frequent both in the leucocytes and in the diplococci. Many leucocytes, even those devoid of diplococci, show nuclear fragmentation and vacuolization, while the markedly degenerated diplococci are commonly intracellular. The number of diplococci in the leucocytes is as variable in the guinea-pig as it is in man. Occasionally one finds that the injected diplococci have undergone agglutination in the peritoneal fluid. Where the injected diplococci were quite uniformly distributed in the salt suspension, cover slip preparations from the inflammatory exudate show the micro-organisms to be in smaller and larger clumps, in which condition they may be taken up by phagocytes.

Since in cerebro-spinal meningitis in man cultivation experiments may fail to yield the diplococcus although the microscope shows it to be present, attention may be called to similar failures with guinea-pigs. In the case of these animals the period of survival after inoculation affects the result, since, if the autopsies are made soon after death, those pigs succumbing earliest give the greater number of successful cultivations. But if the autopsies are delayed and par-

tial decomposition of the body has already set in, or if the dead pigs have been kept for many hours in the refrigerator, the chances of successful cultivation of the diplococcus are greatly reduced. I have noticed that the diplococci taken from pigs dead some hours, which have not grown on planting on a good medium, show irregularities in staining indicative of beginning dissolution; while a pig which had died at about the same time from a corresponding injection of the diplococcus, but which had been kept in the refrigerator during the same number of hours (six to eight), showed the diplococci in a better state of preservation, although they too may not have grown in the tubes. In the first instance, the diplococci have succumbed, probably, to the combined action of inflammatory exudate and the products of decomposition; and in the second, they were probably killed by the low temperature (vide supra). Should the former pigs remain somewhat longer at the fairly warm room temperature, the diplococci may disappear entirely post mortem. Hence, failure to grow upon artificial culture media may depend upon changes suffered by the diplococci in the infected body, or in the body after death. I may anticipate, in this place, an observation made upon the medulla oblongata of a monkey which succumbed to an intra-spinal injection of the diplococcus. The medulla was covered with a recent purulent exudate from which diplococci were easily cultivated. Two portions were excised of which one was placed at 37° C. and the other at 2° C. over night. Neither of these now gave growths, and the cover slip preparations which originally showed numerous diplococci now showed many fewer in the exudate from the section of tissue placed in the thermostat; no such diminution occurred in the cooled tissue.

In view of the manner in which *Diplococcus intracellularis* acts in causing death of guinea-pigs, it may be assumed that its effects are produced by a poison liberated from the bacterial bodies, probably through disintegration, and not to a secreted extracellular poison. Multiplication on the part of the diplococci is not at all essential to the production of the peculiar symptoms and lesions. Even though multiplication of the virulent diplococci does take place, it is still likely that the toxic action is caused by the diplococci which have suffered dissolution. That this is the correct

interpretation is shown by the toxic action (1) of cultures killed by heat, and (2) of the autolyzed fluids—autolysates—of cultures. As regards the autolysates, they can readily be shown to be lethal, by intraperitoneal injection, in mice and small guinea-pigs, of which the latter are the more sensitive. The autolysate prepared by suspending the growth from a pint Blake bottle in 12 c.c. of salt solution will, after filtration through porcelain or centrifugalization till clear, cause death in twenty-four hours in doses of 0.5 to 1.0 c.c. It seems as if the filtered extracts had lost something of their toxicity as compared with the centrifugated ones. Exudates obtained from the peritoneal cavity of guinea-pigs, in which many diplococci had been dissolved, also proved to be toxic for mice. Heating the autolysate to 65° C., at which temperature the enzyme is reduced in activity, does not reduce materially the toxicity; hence, the poison is probably independent of the enzyme. The following tabulation shows the comparative effects of living and dead cultures and auto-

Weight of Pig in Grams.	Substance Injected.	Result.	Post-mortem Appearances.
187	0.5 c.c. susp. living cocci.	Died during night.	8 c.c. turbid fluid in peritoneum; typical lesions. Large numbers of diplococci; some phagocytosis.
150	0.5 c.c. susp. heated to 60° C.	Died during night.	4 c.c. haemoglobin-tinted fluid. Mottled adrenals. Oedema pancreas. No phagocytes or diplococci.
190	1.0 c.c. susp. heated to 60° C.	Survived.	
182	2.0 c.c. susp. heated to 60° C.	Survived.	
260	3.0 c.c. susp. heated to 65° C.	Died during night.	5 c.c. haemoglobin-tinted fluid; typical lesions; multiple haem. in liver; some leucocytes; few fragments; swollen cocci.
152	1.0 c.c. autolysate.	Died during night.	4 c.c. slightly turbid exudate in peritoneum; mottled adrenals; some leucocytes.
182	1.0 c.c. autolysate heated to 65° C.	Died during night.	4 c.c. clear fluid in peritoneum; red adrenals; no leucocytes in peritoneum, but some in omentum.

lysates. All the injections were made into the peritoneal cavity at four P. M.

The fact that this table especially brings out is that heating the diplococci to 60° C. or slightly above reduces their toxicity, while the toxicity of the autolysate is not materially reduced by the temperature. Upon what changes in the diplococci produced by this temperature the loss depends is not shown by this experiment, but perhaps the reduction of enzymotic power, which begins to take place about 60° C. may partially explain the effect. Since the toxic action of the diplococcus depends upon disintegration, reduction in the rapidity of dissolution might easily affect the result. Relation between the weight of the pig, as influencing susceptibility, is also shown to play a part, but one easy to be overcome by increasing the dose of the diplococcus.

In order to ascertain how important viability of the diplococcus is in bringing about the lethal effects a suspension in Ringer's fluid of which the fatal dose had been determined was kept at 2° C. until growth could no longer be obtained. It was assumed that the toxin suffers less injury when the cocci are killed at low than at the higher temperatures. The lethal dose went up from 0.1 c.c. to 0.2 c.c. of the suspension. A second observation bearing on this point was unwittingly made in the course of some experiments with immune sera. A suspension of the diplococcus in Ringer's fluid of which 0.1 c.c. was fatal in 12 to 14 hours in the preliminary test was found, the next day, to be without power to cause death in the control animals and, of course, in the immunized pigs. A large volume of the suspension was transplanted to sheep-serum agar but no growth was obtained. The assumption that multiplication of the injected diplococci takes place during the first hours after inoculation would seem to have more probability than the counter assumption that deterioration of the poison sometimes takes place rapidly from the action of unknown causes.

The most striking lesions in inoculated guinea-pigs occur in the peritoneal cavity. Unless the lethal effect is prolonged beyond one or two days, fluid exudates are obtained, and more or less pus and fibrin cover the anterior surface of the liver, and the surfaces of

the rolled-up omentum. Even with considerable purulent deposit over the liver, the fluid may be nearly clear. The pleural cavities frequently contain an excessive quantity of clear serum, and the lymphatic glands generally are swollen and congested. The main visceral lesions met with are (1) vivid congestion or hæmorrhages of the adrenal glands; (2) hæmorrhages into the mesentery, central tendon of the diaphragm and serosa of the abdominal walls and intestine; (3) gelatinous œdema of the pancreas and peri-pancreatic tissues. A detailed description of these lesions is not merited; but exception may be made of the hæmorrhages in the mesentery. This exception appears justified by the not uncommon occurrence of hæmorrhages into the skin in cerebro-spinal meningitis in man.

The hæmorrhages in the peritoneum in the guinea-pigs are distributed with much regularity. The most common places of occurrence are the mesentery of the ascending colon, below the pancreas, which has an almost circular form derived from the curved loops of intestine, the mesentery of the cæcum, and the central tendon of the diaphragm. Hæmorrhages occur less frequently in other parts of the serous membrane. The mesentery is stretched over bottle-tops, fixed in Zenker's fluid, and stained in hæmatoxylin and eosin. The preparations are thin enough to permit the examination to be made with the one-twelfth inch immersion lens. The hæmorrhages are strictly focal. They arise from capillaries and small veins; and they are common at the point of junction of several veins and capillaries. The extravasation is, usually, from one point of the vessel, but the infiltration of the vascular sheath and adjacent tissue with red corpuscles may be quite extensive. Sometimes only a few corpuscles escape. The vessels from which the blood escapes are not thrombosed; there is often no evidence of marked stasis; judging from the few polymorphonuclear leucocytes adjacent to the escaped corpuscles, inflammation is not a prominent feature. Often no change in the vascular walls at the place of escape of corpuscles can be made out; sometimes the endothelium is massed at the point of apparent escape; rarely is the vessel dilated above or below the point; but in some instances the endothelium has been displaced outwards by the escaping corpuscles.

or even appears to be deficient. The last appearance is similar to that which Flexner and Noguchi[11] described in rattle-snake venom poisoning as caused by "hæmorrhagin," an endotheliolysin contained in that venom.

It has been noted that the toxic autolysate carries all the toxic bodies contained in the diplococci. It is, however, doubtful whether all of them pass equally readily through Berkefeld filters. Larger doses of the filtered "extract" are required than of the centrifugalized ones to produce lethal results; and the exudates caused by the former may not be attended by accumulations of fibrin and pus. Possibly the small fragments of bacterial bodies, not separated by the centrifuge, bring out the richer emigration of leucocytes. Hæmorrhages have, however, been hardly ever noted among the lesions caused by the filtrate.

The great capacity which the body displays for ridding itself of the diplococci, as shown by the enormous numbers of them which disappear from the peritoneal cavity in a few hours, could be readily accounted for by the inherent tendency to self-digestion which the diplococci exhibit. Any mechanism which will either kill the micro-organisms outright, or so reduce their vitality as to bring them under the injurious influence of their own enzyme will suffice to bring about their dissolution. It has already been pointed out that phagocytosis is not essential to the removal of the diplococci from the peritoneum and the reason for this is now clear. It is relatively uncommon to find, in human infections, any number of the diplococci circulating with the blood, and in guinea-pigs, mice and monkeys, the diplococci are present in the blood for a brief relative period only. From these observations the fact might be deduced that the diplococcus does not thrive in the blood serum. David Davis[12] has observed the diplococcus to grow in defibrinated blood of certain normal individuals, but he has also ascertained that normal serum is bactericidal for the diplococcus, and this destructive property is diminished but not wholly removed by heating the serum to 60° C. for thirty minutes. Hence, the question arises whether in the guinea-pig this mechanism of serum-lysis or any

[11] *Univ. Penna. Med. Bulletin*, 1902, xv, 345.
[12] *Jour. of Infectious Diseases*, 1905, ii, 602.

allied phenomenon is employed naturally in removing the diplococci from the peritoneal cavity. An answer to this question may help to explain the bactericidal phenomena which take place in the peritoneal cavity of these animals.

I have made experiments on the dissolving power of the blood-

Two guinea-pigs received intraperitoneal injections of Diplococcus "654" heated previously to 65° C. for thirty minutes. They survived about eight hours. The clear peritoneal exudate was collected: it contained very few leucocytes and many shadowy diplococci. Centrifugalization until clear. Two series of tests were made as follows: fresh guinea-pig serum and the fresh exudate with large and small amounts of living diplococci; serum and exudate heated to 60° C. with the same amounts of living cultures. The tabulation gives the result.

SERIES I.

	Immediate Culture.	Culture after 24 Hours.	Culture after 48 Hours.
Serum 2 c.c. emulsion cocci 0.1 c.c.	Abundant growth.	Reduction in growth.	Many separate colonies.
Serum (60°) 2 c.c. emulsion cocci 0.1 c.c.	Idem.	Idem.	Idem.
Exudate 2 c.c. emulsion cocci 0.1 c.c.	Idem.	No growth.	No growth.
Exudate (60°) 2 c.c. emulsion cocci 0.1 c.c.	Idem.	No growth.	No growth.
	Immediate Cover-slips.	**Cover-slips after 24 Hours.**	**Cover-slips after 48 Hours.**
Serum 2 c.c. emulsion cocci 0.1 c.c.	Many well stained cocci.	Small number of cocci remain.	Increased number of cocci.
Serum (60°) 2 c.c. emulsion cocci 0.1 c.c.	Idem.	Reduction less than previous one; swollen cocci visible.	Greatly increased number of cocci.
Exudate 2 c.c. emulsion cocci 0.1 c.c.	Idem.	Only shadows of cocci remain.	No cocci.
Exudate (60°) 2 c.c. emulsion cocci 0.1 c.c.	Idem.	Only detritus.	Detritus.

Series II was a repetition of Series I, except that 0.5 c.c. of the emulsion of the diplococcus was employed. The striking feature was the complete destruction of the organisms by the unheated exudate; the apparent complete destruction at the end of the first twenty-four hours by the exudate heated to 60° C. and the re-appearance of a small number of colonies from this tube at the end of the second twenty-four-hour period. The serum tubes gave abundant growth in all the transplantations. Cover-slip preparations showed reduction in numbers of diplococci in all the tubes, the reduction being less marked in the tubes of serum than of the exudates.

serum and the peritoneal exudate, from guinea-pigs on the diplococcus. The serum is collected in a sterile manner; the peritoneal exudate is freed from cells by centrifugalization. Both fluids possess digestive power for the diplococci, but in very different degrees. Mixed with fresh cultures considerable numbers of the diplococci can be destroyed and dissolved. In the case of the exudate, the toxicity, as might be expected, is increased by this digestion. The serum and exudate differ not only in respect to the number of diplococci which they destroy in their natural condition, but they also differ as regards the effect of heat on the bactericidal power. Heating to 60° C. for thirty minutes robs the serum of much more of its power to destroy the diplococci than the exudate.

Practically the same result as the preceding has been obtained with exudates derived from guinea-pigs succumbing to living cultures. The power to destroy diplococci is not lost by the exudate after several weeks keeping at 2° C. In the course of the destruction of the diplococcus by the exudate, I have repeatedly noticed the enlarged or swollen, deep-staining diplococci already mentioned of which it has been suggested that they may be more enduring forms of the micro-organism. In safranin-stained preparations these diplococci stand out as sharply defined, highly refractive, deep pink bodies. They are the last morphological elements to disappear from the fluids; and when they can no longer be detected the cultures prepared from the fluids remain, as a rule, sterile. Fresh guinea-pig serum, and to a less extent the heated serum, can destroy all the diplococci added if the number is not too great. There appears to be variations in this power in different normal animals.

The question upon what ingredients the difference in action of serum and inflammatory exudate depends, arises naturally. Perhaps the most important difference in composition would arise from the products of diplococcal disintegration and of leucocyte destruction in the exudate as compared with the serum. We have already seen that the products of diplococcal dissolution are injurious to living diplococci only when they are highly concentrated, and hence it seems as if in the case under consideration they could be left out of account. My own opinion is that the destroyed leucocytes yield to the exudate an injurious substance which acts upon the diplococci

in such a manner as to bring them under the dissolvent action of their own enzyme. Since, also, the fresh and heated (60° C.) exudate is digestive upon diplococci heated to 65° C. and 70° C., it must itself possess dissolvent power. This latter power is probably not complemental but rather proteolytic in nature. Whether the diplococci are subject in high degree to the complemental form of bacteriolysis is not known; but the power exerted by serum heated to 60° C. would rather indicate that another kind of destruction than this ordinarily takes place. What this is can be surmised from the general tendency to dissolution exhibited by the diplococcus. It is necessary only that fresh or heated serum should injure the diplococcus in order that its intracellular bacterial enzymotic power should come into action. In order to explain all the appearances met with in the peritoneal cavity of guinea-pigs infected with *Diplococcus intracellularis,* the various properties of the diplococcus, plasma, and inflammatory exudate, have to be taken into accurate account.

EXPERIMENTAL CEREBRO-SPINAL MENINGITIS IN MONKEYS.

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PLATES II–V.

At the beginning of my study of *Diplococcus intracellularis* I had the plan of using monkeys to reproduce the symptoms and lesions of cerebro-spinal meningitis in man. My study of the intra-peritoneal injections of guinea-pigs encouraged me to believe that the injection of suitable cultures of the intracellularis into the spinal canal of monkeys would lead to the production of an acute inflammation, the symptoms and lesions of which might bear resemblance to those occurring in the natural infection in human subjects. I was not mistaken in this belief. Some of my earliest experiments were made with monkeys. The cultures were introduced into the spinal canal by means of lumbar puncture. The injection was made commonly in the canal at the level of the third lumbar space. This space is not below the level of the cord in many monkeys, but I have never had paralysis follow my inoculations. I endeavored to secure a flow of spinal fluid through the needle before injecting the culture, for, otherwise, doubts whether the injection was carried into the canal or the surrounding muscles might arise. Not infrequently I failed to obtain this flow of fluid even when I felt reasonably certain that the needle was in the canal. In endeavoring to remove this source of doubt I introduced, in a few instances, the needle into the fourth and fifth lumbar spaces, without causing, in a single instance, paralysis. It sometimes happened that fluid could be obtained at these higher levels when it was not secured at the lower ones. Small monkeys, as a rule, seem not to have any considerable quantity of free fluid in the spinal canal. In this respect, this class of animals differs greatly from man. The amount of fluid which is obtainable by lumbar puncture from the monkey

is subject to wide variation in the same species, and in different species. The amount which may be secured from an animal on successive days also differs greatly. In some cases, a few drops may be secured after patient waiting for five or ten minutes; in other cases, the flow sets in immediately and one or two cubic centimeters are rapidly obtained. As will appear below, the quantity of fluid is increased at certain stages of the inflammatory process, to diminish or disappear wholly at others.

In cases in which no spinal fluid has been obtained preceding the injection of the culture, a successful injection can be assumed if the monkey develops symptoms of illness within a few hours of the inoculation, or if a subsequent puncture of the spinal canal yields fluid containing the diplococci and exuded inflammatory cells. I have found that the injection of relatively large quantities of living diplococci into the muscles about the spinal column, or into the muscles and subcutaneous tissues of other parts of the body, produces only trifling effects. There appears at most some local swelling, and the animals may refuse food for a few hours. The tumefaction quickly disappears and the appetite returns. There is a parallel in this respect between the relative susceptibility of the guinea-pig to intraperitoneal inoculation and the high degree of refractoriness which it displays toward subcutaneous injections of the diplococcus. Not a few cultures of the diplococcus have failed to produce symptoms or lesions when injected into the spinal canal of monkeys. Virulence plays an important part in these injections. In some instances the degree of virulence as exhibited in monkeys and guinea-pigs was similar. Cultures which were active against guinea-pigs were active in monkeys; a culture of low virulence for the former was little pathogenic for the latter animals. This parallelism was well shown by a culture "Smith," which retained its virulence through several months of artificial cultivation. When its power to infect guinea-pigs was finally greatly diminished, it had lost to a large degree its power to cause serious lesions in monkeys. In the experiments upon monkeys to be related, suitable cultures for inoculation were, as a rule, chosen by testing them in advance upon small guinea-pigs.

At the time my experiments were begun there was, as far as I

was aware, only one report in the literature upon the use of monkeys to produce cerebro-spinal meningitis by injecting cultures of *Diplococcus intracellularis* into the central nervous system. Bettencourt and Franca[1] attempted without success to infect monkeys. They trephined one monkey and introduced the culture beneath the dura mater; they injected in two other monkeys cultures into the spinal canal. In still another monkey they attempted to infect the animal by rubbing the nasal mucous membrane with cotton moistened with a culture of the intracellularis. The successful experiments of Von Lingelsheim and Leuchs[2] were published after my first communication on this subject had appeared.[3] Weichselbaum[4] succeeded by subdural inoculation in three instances in dogs in producing acute pachy- and leptomeningitis associated with encephalitis; Councilman, Mallory and Wright[5] successfully produced acute meningitis in a goat by injecting a bouillon suspension of the diplococcus into the spinal canal. Albrecht and Ghon,[6] and Bettencourt and Franca[7] failed to confirm this result in their experiments upon goats.

The monkey offered itself as a suitable animal for the inoculation experiments because of its relationship to man; the fact of its upright carriage, which would permit comparison with man in respect to the distribution of the lesions, and because of the ease and safety with which repeated lumbar puncture could be made in following the course of the disease. The protocols of certain successful experiments will be given.

Monkey No. 1. Macacus Nemestrinus.—April 12, 1905, 10:30 A. M. A moderately large monkey was etherized,[8] after which a needle was introduced into

[1] Uber die Meningitis Cerebro-spinalis Epidemica und ihren specifischen Erreger. *Zeit. f. Hyg. u. Infectionskrank.*, 1904, xlvi, 463.

[2] Tierversache mit dem Diplococcus intracellularis (Meinigococcus), *Klin. Jahrbuch*, 1906, xv, 489.

[3] Experimental Cerebro-spinal Meningitis and its Serum Treatment, *Jour. Amer. Med. Assoc.*, 1906, xlvii, 560.

[4] *Fortschritte der Medicin*, 1887, v, 622.

[5] Epidemic Cerebro-spinal Meningitis and its relation to other forms of Meningitis, Boston, 1898.

[6] *Wien. klin. Woch.*, 1901, xiv, 984.

[7] *Op. cit.*, page 500.

[8] In later experiments ether was not used, as the operation gave little pain.

the spinal canal. Clear fluid escaped from the needle. A culture eighteen hours old of *Diplococcus intracellularis*, in its first generations on Loeffler's serum, suspended in salt solution, was injected. The monkey quickly recovered from the operation. Six hours later it appeared to be sick; at 6 P. M., it no longer sat on its perch; 9 P. M., still on bottom of cage, very sick, head down. On being disturbed, it moved slowly. Death during the night. Survived injection probably eighteen to twenty hours.

Autopsy 9 A. M., April 13. The internal organs, except the nervous system, show no striking lesions. Brain and Spinal Cord.—No marked excess of fluid in the meninges. The convex surface of the brain is greatly congested, and punctiform hæmorrhages exist in the pia-arachnoid and the superficial portions of the cortex. No definite exudate can be made out on the convex surface of the convolutions, although the pia of the sulci appears white and is slightly thickened (probably an old condition). A focus of softening or an abscess, the size of a large pea, occurs to the right of the superior longitudinal sinus in the convolutions anterior to the motor area, and just beneath the membranes. The base of the brain is covered with an opaque exudate, white in color, which extends over the medulla and, anteriorly, to the optic commissure. The lumbar and thoracic portions of the spinal cord are covered, chiefly posteriorly, with an opaque white exudate. The cerebral ventricles contain an increased amount of fluid of turbid appearance.

The diplococcus was recovered in cultures from the exudates of the spinal cord and base of the brain, and from the blood of the heart. Cover-glass preparations from the exudates show that (1) the pia of the convexity contains many polymorphonuclear leucocytes and few diplococci; (2) the pia covering the medulla contains large numbers of leucocytes and intracellular cocci, and a small number of extra-cellular cocci; (3) the ventricles contain desquamated ependymal epithelium, many leucocytes, and few diplococci; and (4) the focal lesion in the convexity of the brain consists of a collection of polymorphonuclear leucocytes containing very large numbers of typical diplococci. Few or no diplococci occur outside of cells in this focus (Plate II, Fig. 1).

Sections of the brain and cord show an abundant emigration of polymorphonuclear leucocytes into the leptomeninges chiefly. The pia-arachnoid of the convexity contains a richer exudate than was evident to the naked eye; but the exudate covering the base of the brain is present in much the greater amount. It consists chiefly of leucocytes; fibrin occurs in very fine strands and in small amount only. The ventricular fluid contains many leucocytes and no fibrin. A striking feature of the inflammation is the invasion from the pia-arachnoid of the superficial portion of the cortex directly, and of removed parts at considerable depths indirectly along the sheaths of the blood vessels. The vascular sheaths and the perivascular lymphatic spaces are richly infiltrated with polymorphonuclear leucocytes from which locations the surrounding brain tissue is being invaded by emigrating leucocytes. Sections through the filum terminale show the leucocytes to surround the cord in the pia, a slight invasion along the septa into the nervous tissue, and thick perivascular emigration about the deepest vessels of the dura mater.

This successful experiments was obtained by the use of a recent

culture isolated from the spinal fluid of a child "Kepp." The fluid was quite opaque from the many leucocytes present and it contained a moderate number of the diplococcus, both within leucocytes and free in the fluid. A second monkey of the same species and about the same size as the first was inoculated with one cubic centimeter of the sedimented exudate. Immediately after the injection, the monkey became rigid and the head was retracted; recovery took place in a short time. In spite of the fact that no fluid escaped from the needle, no doubt was entertained that the injection entered the spinal canal. However, no symptoms of disease developed from this injection.

Monkey No. 3. Small Macacus rhesus.—April 15, 1905, 9. A. M. Inoculated by lumbar puncture with two loops suspended in salt solution of "Bingley" culture of the diplococcus from spinal fluid. 5 P. M., animal sick; sits on bottom of cage. 9 P. M., very sick; crouches on bottom of cage with head depressed; moves slowly on being disturbed. April 16, 9 A. M., still alive; head down almost to level of the floor; a little later, the monkey is lying on one side and is passing through a convulsion. The convulsive seizures follow each other at short intervals and are excited by sudden noise or by contact. 3 P. M., convulsions still occurring; lumbar puncture yields a small quantity of bloody fluid which on microscopical examination shows many polynuclear leucocytes containing typical diplococci. 10 P. M., convulsions continue. Animal died during the night; probably survived about forty-three hours.

Autopsy, 9 A. M., April 17. No visible lesions of the internal organs except the central nervous system. The convex surface of the brain shows great injection of the pial vessels; and the pia-arachnoid contains a gelatinous fluid exudate. Purulent exudate is visible surrounding the infundibulum only. The fluid in the ventricles is slightly increased. No visible exudate covers the spinal cord. The cultures made from different portions of the brain and cord remained sterile. Sections of the spinal cord show a small quantity of purulent exudate in the meninges; those from the brain show a richer leucocytic infiltration of the pia-arachnoid, and the invasion of the choroid plexus of the lateral ventricles. The meninges of the sulci are especially infiltrated. From the superficial meninges, and from the intra-cortical vessels, the brain substance has been infiltrated with many leucocytes.

As regards this experiment I wish to point out that when the inoculated monkey survives through the second day, the quantity of exudate may not be very considerable, and the diplococci, if still present, may fail to grow on a suitable culture medium.

Monkey No. 7. South American; Genus Cebus.—May 19, 1905, 3 P. M. One cubic centimeter of a suspension of spinal fluid culture "Goldman" injected. This South American species is considerably smaller than the Macacus monkeys employed. It was found dead at 7 A. M., May 20, but evidently had been dead

only a short time. Probably survived fifteen hours. The autopsy showed a small amount of exudate over the spinal cord in the lumbar region, turbid fluid along the base of the brain to the optic commissure, vivid injection of the pia of the convexity, and an increased quantity of turbid fluid in the cerebral ventricles. Cultures were positive. Smear preparations from different parts of the brain and cord show the leucocytic exudate to be general, and a fairly large number of diplococci, chiefly intracellular, to be present. Many of the diplococci within leucocytes are swollen or otherwise degenerating. The preparations made by smearing bits of the nasal mucosa on slides show a considerable number of polymorphonuclear leucocytes among the high epithelium and mucus. *A small number of the leucocytes contain many diplococci morphologically like those of the exudate in the brain, and presenting the same degenerations.*

A study of the brain and cord in sections shows the inflammatory lesions over the convexity to be more pronounced than was evident to the naked eye. The pia is everywhere invaded by polynuclear cells, and the exudations form thick, wedge-shaped infiltrations between the convolutions. A fine network of fibrin unites the leucocytes. The invasion of the cortex is a marked feature of the lesions. The leucocytes have passed into the brain tissue from the surface and along the sheaths of the vessels (Plate II, Fig. 2). An intermediate zone of non-infiltrated tissue exists between the two layers. The invasion of leucocytes into the brain stops rather abruptly a centimeter or so from the surface, and the deeper vascular sheaths are devoid of these cells. Among the leucocytes which have emigrated into the cerebral tissue are a small number of eosinophilic cells. A section through the medulla shows less exudate than some parts of the cortex. The deeper parts of the medulla have also been invaded from the vascular sheaths. The lateral ventricle has been invaded along the choroid plexus, and an accumulation of leucocytes occurs below the epithelium, which is partly deficient, at one side of the ventricle. The thoracic region of the spinal cord is relatively free from exudate.

A spinal root ganglion included in a section of the spinal cord is surrounded, beneath its fibrous capsule, by a collar of leucocytes which are penetrating among the nerve cells (Plate III, Fig. 3). Sections of the optic nerve show leucocytic invasion. Diplococci are numerous in the leucocytes in the pia-arachnoid. They cannot be found with certainty in the leucocytes or free in the brain tissue.

This experiment serves to show that in a brief period of fourteen or fifteen hours advanced and deep lesions can be produced in the brain and its membranes by *Diplococcus intracellularis*. The experiment also indicates that the inflammatory reaction may be more active in the brain than in the spinal cord. The inflammatory exudate resembles in character that present in acute cases of epidemic meningitis in human subjects. Emphasis should, perhaps, be laid upon the findings in the nose and in the spinal ganglion. It is usual to find in the monkeys succumbing to the experimental infections, evidences of inflammation of the nasal mucosa. Smear preparations from the superior mucous membrane often show a

variable number of leucocytes enclosing diplococci presenting the morphology and staining properties and the degenerations of the diplococci in the brain and cord. The inflammation and purulent infiltration of the spinal ganglion is interesting in relation to the similar finding in cases of cerebro-spinal meningitis in man by Councilman, Mallory and Wright.[9]

Monkey No. 10. Macacus rhesus.—May 26, 1905. At 11 A. M. one half agar culture "Cohn" suspended in salt solution was injected into the spinal canal. Fluid escaped from the inserted needle. 5 P. M., animal very sick; living at 9 P. M. Found dead at 6 A. M., May 27. Probably survived fifteen hours.

Autopsy: The meninges of the inoculated portion of the cord show minute hæmorrhages and cloudy exudate. The meninges of the medulla contain an increased quantity of cloudy fluid. The pia covering the convex surface of the brain is hyperæmic, especially over the occipital lobes, which present an almost uniformly reddish tint; while the pia and the adjacent brain substance are everywhere beset with minute hæmorrhages. Cultures from the lumbar portion of the cord, third ventricle, and pia over the medulla all give pure growths of the diplococcus. No growths are obtained from the cortical meninges and the lateral ventricle. The furthermost portion of the dura mater, extending beyond the olfactory lobes into the nose, is covered with an inflammatory exudate in which polymorphonuclear leucocytes carrying biscuit shaped Gram-negative diplococci exactly resembling in form, preservation and staining, those present in the meninges of the brain and cord occur. Smear-preparations from the turbinated bones show very few leucocytes and no diplococci. Cultures made from the mucus of the turbinated mucous membrane did not yield the diplococcus.

The sections of the brain show a moderate inflammatory œdema affecting the pia-arachnoid of the base and the convexity. The especial pathological conditions to be emphasized are (*a*) the great congestion of the pial blood-vessels and the extravasation of red corpuscles; (*b*) the direct inflammatory invasion of the superficial cortex from the pia which is associated with rarification of the brain tissue; (*c*) the profound changes in the blood-vessels of the brain proper and the degeneration and leucocytic invasion of the surrounding brain tissue (Plate III, Fig. 4). In some parts of the cortex, adjacent to the sulci, the leucocytic accumulation is so rich as to simulate abscess formation; no softening has, however, taken place. The emigration of leucocytes from the intracortical vessels was active at the time of fixation of the tissues. The perivascular lymphatics contain fibrin and coalesced red-corpuscles, and the blood-vessels themselves are occluded, in places, by coalesced (agglutinated) red corpuscular and by fibrinous thrombi. The punctiform hemorrhages in the brain tissue arise from these injured and occluded vessels. *Sections of the dura mater extending beyond the olfactory lobes show a richer accumulation of leucocytes than elsewhere in the membrane itself and an invasion of the substance of the membrane.*

Typical diplococci occur within leucocytes in the meningeal exudation and

[9] *Op. cit.*, p. 114.

they are moderately numerous. They exist also in the leucocytes in the brain tissue, adjacent to the blood-vessels from which the leucocytes have emigrated. No diplococci are found in the brain tissue outside of cells.

A lengthy discussion of this experiment is not called for since it carries its own explanation. Attention may, perhaps, be directed again to the co-incident effects produced by the diplococcus upon the membranes and the nervous tissues. The wide involvement of the blood vessels is a significant fact in the pathology of this experimental disease; and the evidence of diffusion of the poison from the blood vessels of the brain or from the peri-vascular lymphatics into the brain tissue is made strong by this experiment. The agglutinative red corpuscular thrombi constitute a novel feature of the pathological condition. The rich assemblage of leucocytes surrounding the olfactory lobes and extending into the substance of the dura mater seems to me not wholly without significance in view of the possibility of infection of the nasal passages with diplococci from the brain.

In view of the importance of this condition for explaining the appearance at times, of *Diplococcus intracellularis* in the nasal and pharyngeal cavities in human subjects of cerebro-spinal meningitis, I gave especial attention to the study of the olfactory nerves and the nasal passages in several monkeys. It may, in the first place, be stated that the turbinate and septal mucous membrane will be found to be vividly congested in all the animals which succumb to the acute infection. If smear preparations from the mucous covering the inflamed membranes, or, better, from bits of the mucous membrane snipped off with scissors be examined, a variable but increased number of polynuclear leucocytes will be seen. If the leucocytes are compared with those present in the meninges about the base of the brain, they will be found in the same condition of preservation or degeneration as the latter. Moreover, in those cases in which diplococci are still demonstrable in the leucocytes in the brain, a certain number, sometimes relatively many, of the leucocytes in the nose contain Gram-negative diplococci resembling in all external features the former micro-organisms. If the dura mater beginning at the olfactory bulbs, surrounding the olfactory nerves, and extending through the cribriform plate into the nose, be carefully removed with the adjacent

portion of the ethmoid bone and olfactory mucous membrane, fixed in Zenker's fluid, sectioned longitudinally, stained in hæmatoxylin or methylene blue and eosin, and examined microscopically, the passage of leucocytes from the brain cavity into this membrane and about the olfactory nerves towards the ethmoid, can be traced. The blood-vessels of the adjacent olfactory mucous membrane are seen to be dilated and leucocytes to be passing into the layer of columnar epithelium of the surface.

The abundant lymphatics of the mucous membrane are in communication with the lymphatic spaces which enclose the branches of the olfactory nerves, and these spaces again communicate with the subdural and subarachnoid spaces of the cranium, so that the lymphatics of the nasal mucous membrane can be injected, according to Schwalbe and Key and Retzius, from the cranial cavity.

I have not succeeded, although I have made several attempts, in cultivating *Diplococcus intracellularis* from the nasal mucosa of these monkeys. I realize, of course, that it is highly desirable and very important to bring this final proof of the passage of the diplococci from the cranial cavity into the nose. The difficulties which surround the demonstration are considerable, for I think it probable that the diplococci do not long remain viable in the nose in monkeys. I conceive the conditions there to be, possibly, even more unfavorable to them than in the meninges where, indeed, they tend rather to disintegrate than to multiply. The conditions in man are, of course, very different and far more favorable to the existance of the diplococci. But in spite of the incompleteness of the proof in the case of the monkeys, I would still urge that attention be given to this possible mode of infection of the nasal and pharyngeal passages in human subjects.

The next experiment to be described brings out a different point, namely, that of a fatal issue, after the lapse of thirty hours, with which is associated the gradual diminution and degeneration of the diplococcus, without any marked participation of leucocytes in the process, or production of striking pathological lesions. It recalls forcibly certain observations made on guinea-pigs.

Monkey No. 12. South American; Genus Cebus.—June 1, 1905, at 11 A. M., a small monkey was inoculated with one half agar slant culture "Smith"; fluid

flowed from the needle before injection. 12 M., lumbar puncture (= l. p.) 1 c.c. almost pure blood obtained; it showed on cover-slips (= c. s.) many free diplococci. 3:15 P. M., l. p. small quantity blood secured; c. s. many cocci free and very few in leucocytes. Animal not sick. 9 P. M., l. p. a few drops of slightly blood-tinted fluid obtained; c. s. many free diplococci and a small, although increased number of leucocytes containing diplococci. Animal less lively than before. June 2, 9 A. M., monkey is very sick; temperature subnormal; head droops below arms; no convulsions on disturbance; l. p. a few drops of blood-tinted fluid secured; c. s. leucocytes increased, some containing diplococci part of which stain feebly; many free diplococci. 3 P. M., l. p. leucocytes still not numerous; intracellular and extracellular diplococci present, but the number is diminishing. Cultures from the fluids obtained at the punctures gave growths in every instance. About 4 P. M. the monkey showed general weakness and retraction of the head. Dr. Meltzer, who examined the animal, pronounced the latter condition not to be opisthotonus. Death at 5 P. M. On ice till next day.

Autopsy, June 3, 9 A. M. Neither the cord nor the brain show any marked lesions.

The pia of the cord is slightly injected; a small quantity of clear fluid is present in the pia of the medulla; the intraventricular fluid may be slightly increased. Cultures are negative from brain and cord and the heart.

Smear preparations from the meninges of cord and brain and the fluid of the ventricles show a small or moderate number of diplococci lying free, and almost no leucocytes whatever except in the spinal cord at the point of inoculation. A moderate number of the cells containing diplococci, many of which are degenerating, occur there. Elsewhere it is exceptional to find polynuclear cells containing diplococci. The failure of the diplococcus to grow in cultures made at autopsy does not signify, since the power to grow may be quickly lost at the temperature of the refrigerator. Smear preparations from the nose show no typical diplococci, while a few typical organisms are present in the smears from the pia about the olfactory lobes. The microscopical examination of sections of the spinal cord and brain bear out the naked eye appearances. The membranes show a slight but definite accumulation of leucocytes; there is no œdema, and the nervous tissue is free from appearances of degeneration or from leucocytic invasion. The leucocytes are, however, increased in numbers in the cortical vessels.

The next experiment records an instance of fatal effects, from a relatively small dose of the diplococcus, which was delayed until about sixty hours after injection. The gross lesions were slight; the microscopical ones were definite and interesting because the period of infection was long enough to permit of reaction on part of the fixed tissue cells.

Monkey No. 18. Macacus rhesus.—June 24, 1905, 10 A. M., injected one third culture "Smith." Symptoms of illness developed in the usual time and were pronounced during the first twenty-four-hours. June 25, 12 M., lumbar puncture. The fluid obtained showed many leucocytes, but no diplococci. Cul-

ture negative. The animal did not fully recover and died about sixty hours after inoculation. The autopsy revealed congestion of the meninges, and a small excess of turbid fluid in the lateral ventricles. Cultures on sheep-serum agar were made from several parts of the nervous system. Growths were obtained on the second day, starting from the condensation water, in the tubes inoculated from the third ventricle and lumbar cord. Sections from the brain and cord show, first, a marked congestion of the veins which is greater in the meninges of the brain than of the cord. Second, a small degree of leucocytic exudation throughout the meninges, which in the brain is more abundant in the sulci than over the convolutions. But the most striking and, perhaps, important feature is the great increase of large monoculear pial or connective-tissue cells which by reason of their number make up a large part of the exudate. These cells are larger than leucocytes, possess single vesicular nuclei excentrically placed, and pale and transparent protoplasm.

To be contrasted with the previous experiment is the next one in which the inoculated monkey survived hardly more than twelve hours, in the course of which period marked gross lesions appeared in the nervous system.

Monkey No. 17. South American; Genus Cebus.—June 22, 1905, 10 A. M., injected one half agar culture "Smith," twenty-four hours old. Fluid flowed from needle before injection. 9 P. M., monkey dying. Found dead at 6 A. M., June 23. The autopsy showed the entire spinal cord congested and infiltrated with turbid fluid. In the cerebral meninges smaller and larger hæmorrhagic foci were visible. The pia of the pons and medulla was vividly congested, and a fluid exudate occupied the meshes of the pia-arachnoid generally, and was most abundant over the medulla. The ventricles were unchanged, apparently. Cultures gave the following result: Abundant growth of the diplococcus from the lumbar cord and base of brain; a few discrete colonies from the cortex; no growth from the lateral ventricle. Smear preparations bore out the naked eye indications of the distribution of exudate and diplococci. Sections of the tissues show marked leucocytic infiltration of the membranes of the cord and brain, and a greater accumulation of cells in the basal membranes and the membranes of the sulci. The infiltration is wholly polymorpho-leucocytic. The most striking lesion, however, is the hæmorrhage which is focalized in the membranes and in the superficial brain matter—in the basal and cortical parts. The hæmorrhages in the brain tissue proper have arisen (*a*) by direct extension from the menigeal extravasations, and (*b*) from the vascular branches within the substance of the organ itself. They form elongated or circular foci, depending on the direction of the section. Another lesion of interest is an acute endarteritis which affects the larger and smaller arteries, chiefly at the base of the brain. A sub-intimal infiltration of cells consisting of polymorphonuclear leucocytes and mono-nuclear cells is seen. The vessels are rarely wholly or almost occluded by this accumulation of cells beneath the intact endothelium. Acute encephalitis was a prominent feature of this case.

This experiment, taken into account with others to be described, tends to show that the diplococci rise in the spinal canal to the

medulla, spread themselves over the base of the brain, extend into the cortical meninges, and lastly enter the ventricles. The inflammatory lesions follow in the wake of this extension and doubtless are directly due to the presence of the diplococcus. Attention should perhaps be directed to the hæmorrhages; and to the acute endarteritis mentioned in this protocol of which other examples will be described.

Monkey No. 25. Macacus rhesus.—June 21, 1906, 11 A. M. Inoculated with one agar culture "596." Fluid flowed from needle before injection. 2 P. M., animal appeared sick; lay on bottom of cage, but on being disturbed sat up. 6 P. M., depression increased. 12 P. M., still lying on bottom of cage, but not prone and rises to sitting posture when disturbed. June 28, 8 A. M., brighter; sits up. 12 M., attempted lumbar puncture. Animal resisted with considerable vigor making the puncture difficult. The struggles brought on sudden collapse from which partial recovery took place. Monkey lay on bottom of cage breathing very rapidly; drank water. 2 P. M., still very sick. From this time until 3:45 P. M., when death occurred the animal became weaker and developed intermittent convulsions. The autopsy showed no general visceral lesions. On exposing the spinal cord, it was found congested and covered with a thin, whitish exudate which was more abundant in the lumbar region than in the thoracic and cervical regions. The blood-vessels of the dura mater were injected; but the turgid pia vessels showed through this membrane. On removing the dura, the pia-arachnoid was seen to be vividly injected, the congestion affecting the main and smaller vascular branches and producing a remarkable picture of hyperæmia. There was no visible exudate over the convex surface of the brain. At the base, extending from medulla to optic commissure, there was a turbid fluid exudate. The ventricles contained turbid fluid, but were not dilated. The basal exudate followed the dura over the olfactory lobes into the ethmoid plate. The nasal mucosa was congested. Cover-slips showed the following: From the lumbar spinal cord many leucocytes and diplococci, the latter both free and in cells; from the thoracic and cervical regions of the cord, many leucocytes and fewer cocci; from the convexity—anterior and posterior—impression preparations indicated that few or no leucocytes collected in this region, and the diplococcus was almost wholly absent; from the medulla many leucocytes and extra- and intracellular diplococci; from the ventricles leucocytes and diplococci in small numbers; from the deepest part of the dura at the ethmoid bone many polynuclear leucocytes, but no typical examples of the diplococcus. Cultures from all the sources remained sterile. Since more than two days elapsed, owing to accidental circumstances, between the death of the monkey and the autopsy, it may well be that the failure of cultures was due to this cause. The same condition is capable of diminishing the number of diplococci which can be made out in smear-preparations. But the distribution of emigrated leucocytes showed clearly where the infection was severe, and where it was in its beginning stages. The sections of the tissues bear out the gross findings. They show the existence of great congestion of the pial veins, a small degree of leucocytic infiltration of the pia covering the con-

volutions, and somewhat greater accumulation of inflammatory cells in the sulci. The pia of the deeper parts of the sulci is hardly more than œdematous. The brain substance shows no leucocytic invasion, and the veins do not carry any marked excess of leucocytes. The lower spinal cord shows rather more emigration of leucocytes than the brain. The exudate is collected on the surface of the cord, upon and between the nerve roots (Plate IV, Fig. 5). Fibrin occurs as fine strands and in small quantity.

This experiment brings out several points, some of which have not been especially emphasized before. The effects of muscular exertion on the progress and termination of the infection should, perhaps, be remarked. Since in no other experiment was so great a degree of vascular congestion noted, I should be inclined to view this condition as one of the striking consequences of the muscular over-exertion. It is a short step from this assumption to supposing the marked œdema of the pia in the sulci to have followed the congestion. This view is rather borne out by the poverty of the œdematous fluid in emigrated cells. It has been pointed out on the basis of some of the earlier experiments, and the same fact is indicated by this experiment, that the injected diplococci tend, regularly, to be distributed upwards along the spinal cord and the base of the brain. The invasion of the pia of the convex and mesial surfaces of the brain takes place at a later period, and the infection of the ventricles also takes place later, either because the diplococci are delayed in reaching them, or because they resist infection longer. There is, moreover, relation between localization of the diplococci and the degree and extent of the exudation. Where the accumulation of inflammatory products is most marked the diplococci will, if still present at all, be most abundant. Finally, the growth of the diplococcus upon a suitable culture medium will be greatly influenced, if not wholly determined, by the conditions to which the infected body is subjected after death. While a temperature just above the freezing point will preserve the morphological elements of the body and even the body of the diplococcus, yet if it is maintained for many hours, it may render the diplococcus incapable of multiplication. Higher temperatures subject the body not only to autolytic and putrefactive processes which interfere with the pathological examination, but they deprive even more quickly than cold the diplococcus of power of growth and, under certain conditions, of staining properties as well.

The temperature at which the body is kept, and the elapsed period of time between death and the examination, have a direct bearing upon the cultivation of *Diplococcus intracellularis* from the infected body. I am of the opinion that these factors operate in much the same way in cases of human meningitis as in the case of the artificially infected monkeys and guinea-pigs. Certain discrepancies in the bacteriological studies of cases of meningitis in man may, in view of these considerations, receive an explanation.[10]

On account of its bearing upon this subject and as a transition to the next topic to be discussed, an experiment in which the influence of two extremes of temperature upon the diplococcus was tested, will be introduced here.

Monkey No. 36. Macacus rhesus.—October 24, 1906, 3:30 P. M. After clear fluid was obtained, one cubic centimeter of a turbid suspension of Culture "654" was injected. The injected quantity was the equivalent of two agar slants; the culture had proved itself of relatively low virulence for guinea-pigs. October 25, 10 A. M., monkey sick; sits on perch holding head in hands; easily disturbed. L. p. no fluid obtained; needle-point covered with exudate consisting of leucocytes crowded with diplococci staining sharply. The nuclei of a small number of cells contained cocci. October 26, 12 M., monkey brighter and more active. L. p. slightly cloudy fluid flowed at once from the needle. C. s. showed leucocytes in good numbers, some of which contained few and others very large numbers of cocci staining feebly. Many of the leucocytes had probably recently emigrated since they were of normal appearance with circular (horse-shoe), non-fragmented nuclei. October 27, monkey recovered. November 10, animal in excellent condition.

November 14, 4 P. M., l. p. gave fluid readily; it flowed from needle as if under some pressure. One cubic centimeter of suspension of coccus "596" injected. No immediate effect produced. 6 P. M., monkey less lively. November 15, 7 A. M., found dead; evidently died only a short time before. Autopsy. The spinal membranes were distended and on incision a small quantity of turbid fluid escaped. The cord appeared pale, probably because of the turbid exudate which covered it. The convex surfaces of the brain were moist; definite inflammatory exudate was not visible; the basal membranes, from the medulla to the optic commissure, were infiltrated and distended with a turbid exudate. The pituitary body was surrounded with exudate; the ventricles were not widely dilated. The nasal mucosa was pale. Of the other organs only the lungs showed a pathological condition, namely, congestion and œdema. Cultures from the brain and cord, made at 4 P. M., gave growths of the diplococcus; the heart and lungs gave no growth. At 5 P. M. two segments of the medulla, about 0.5 centimeter thick,

[10] Westenhoeffer, Pathologisch-anatomische Ergebnisse der oberschlischen Genickstarreepidemie von 1905, *Klinisches Jahrbuch*, 1906, xv, 447.

were placed in sterile glass dishes which were enclosed in second dishes filled with cotton saturated with water. One was put at 37° C., the other at 2° C. At 10 A. M. next day, cover-slips and cultures were made; the latter gave no growth. C. s. showed the diplococci to have disappeared almost entirely from the tissue kept in the thermostat, the few remaining cocci being pale and ill-staining; and the diplococci from the second piece of tissue kept at 2° C. to be, perhaps, somewhat less numerous than in the control, but to stain sharply and well.

Histological examination of the central nervous system establishes the existence of a severe acute fibrino-purulent inflammation of the meninges of the cord and brain. The exudate in the cord is thicker over the posterior surface and it surrounds all the nerve roots; the dura mater is also invaded with pus cells. The exudate covering the medulla is abundant, while that covering the cortical surfaces is slighter in amount. The sulci show more exudate than the convexities. Two appearances should be emphasized: the great proliferation of endothelial cells of the pia, which cells mingle with the polynuclear leucocytes; and the diplococci, which are abundant in the exudate of the cord and medulla, innumerable in the exudate about the pituitary body and sparse in the exudate of the cortex and ventricles. The diplococci are, excepting those about the pituitary body, almost exclusively intracellular; and although a slight grade of encephalitis occurs, very few diplococci can be discovered in the leucocytes in the brain tissue. Thrombi of leucocytes occlude many veins.

The experiments reported thus far would seem to establish conclusively that the lesions and, to a certain extent, the symptoms of acute meningitis as they occur in man can be reproduced experimentally in monkeys. Since in the course of the experiments, the symptoms during life were observed incidentally only, the striking ones alone were recorded. Attention should be directed to the occurrence of nystagmus in a rhesus monkey which succumbed to inoculation. It should be stated, also, that not all the monkeys inoculated into the spinal canal succumb to the injections, next that after apparent recovery death may take place suddenly from causes which the post-mortem examination may fail to reveal, and finally that subcutaneous and intramuscular injections of large quantities of the diplococcus cause slight or no evident symptoms of disease. The cases of recovery will be illustrated by a few protocols. It should be noted that as recovery proceeds spinal puncture may show increasing cellular exudation into the canal and progressive degeneration of the diplococci. Cultures from the exudate at first positive become negative before all the diplococci disappear from it.

Monkey No. 5. Macacus nemestrinus.—May 15, 1905. This animal had been injected, April 12, 1905, with a purulent exudate without result (page 146). At

4:30 P. M. one cubic centimeter of a turbid suspension in salt solution of a diplococcus in its fifth generation from the spinal canal of case "Behren" was injected into the spinal canal. No symptoms had appeared by 9:30 P. M. May 16, 8 A. M., animal refused food and appeared sick. 11 A. M., had left perch and sat on bottom of cage. Appeared unable to lift himself on to the perch; while being observed two convulsions occured. Placed on the perch, he held on with hands and feet and was in a state of unstable equilibrium. Made no effort to escape from the cage on being handled. 10 A. M., lumbar puncture; a small amount of bloody fluid containing a moderate number of leucocytes and extra- and intracellular diplococci obtained. 3:30 P. M., l. p.: a small quantity of purulent fluid secured. The greater number of cocci were within leucocytes, many of which showed fragmented nuclei. Cultures from the exudate positive for the diplococcus. 6 P. M., back on perch. May 17, 8 A. M., again on bottom of cage; looked very sick, but took a little milk. 10 A. M., l. p.: the number of diplococci had diminished; very few were outside of cells; those in leucocytes stained feebly. May 18, 10 A. M., l. p.: a few drops of blood-tinted fluid secured; c. s.: some polymorphonuclear leucocytes, increasing number of mononuclear leucocytes, and a very small number of leucocytes containing degenerated diplococci; a few extracellular diplococci still present. Cultures negative. This monkey recovered rapidly.

Monkeys No. 19 and 20. Macacus rhesus.—June 27, 1905, 10 A. M. Each of these monkeys was injected with one third culture "Smith." They became very sick during the afternoon, and lay on the bottom of the cage. 10 P. M., still very sick. June 28, 9 A. M., both animals looked brighter. 10 P. M., No. 20 seemed livelier than No. 19. June 28, 12 M., l. p.: both yielded a small quantity of turbid fluid. The fluid from No. 19 and No. 20 contained many leucocytes; the fluid from No. 19 contained typical intracellular diplococci in small numbers, while that from No. 20 contained none. A culture was made from each fluid: No. 19 gave one colony of the diplococcus, No. 20 gave none. Complete recovery quickly followed in both instances.

Monkey No. 35. Macacus rhesus.—July 10, 1906, 10 A. M. Given 1 c.c. of thin suspension of Diplococcus "596." Spinal fluid obtained with syringe. The only symptoms which developed were slight depression, and erection of the hair covering the body. July 11, 10 A. M., l. p.: a small amount of whitish fluid from which the cells quickly subsided, was obtained. C. s. showed a large number of agglomerated, polymorphonuclear leucocytes, and very few diplococci, chiefly contained in the cells. The few free cocci could easily have come from broken-up leucocytes. At this time, a second injection of 0.5 c.c. of a similar suspension of Diplococcus "596" was made; no result. Animal quickly recovered.

To insure a fatal outcome a diplococcus of known virulence in a sufficient dose must be injected. Individual differences in susceptibility occurred among the monkeys, but no monkey was wholly refractory to inoculation. As compared with the doses which probably determine infection in man those used to produce the experimental disease in monkeys are colossal. As an example of

refractoriness the next experiment will serve; it will show also how small the effect produced by subcutaneous and intramuscular injections of the diplococcus may be.

Monkey No. 26. Macacus rhesus.—October 28, 1905, 12:30 P. M. One "Smith" culture injected. 3:30 P. M., l. p.: turbid fluid flowed from the needle. C. s. showed many diplococci and very few leucocytes. Some of the cocci grouped about the leucocytes. October 29, 12 M., l. p. not successful; one half culture "Smith" injected (probably into muscles of back). October 30, 3:30 P. M., l. p. unsuccessful. Injected two cultures "Smith" (probably into muscles of the back). 6 P. M., animal slightly depressed; off perch. October 31, A. M., animal active. 3 P. M., l. p. yielded with difficulty a small amount of bloody fluid containing leucocytes and few diplococci. Injected three cultures "Smith"; no effect. This monkey showed remarkably little depression following the injections, and was soon in a normal condition.

It is somewhat remarkable that the later injections failed to influence the result of the first injection, which evidently reached the spinal canal. I have observed many other examples of the relative innocuousness of the diplococcus when introduced beneath the skin and into the muscles. The effects of large doses given in this way may be nil, or a slight elevation of temperature may appear and remain for a few hours, or the animal show for a brief period disinclination for food. The next experiment is introduced as an example of sudden death, after apparent recovery from an inoculation.

Monkey No. 21. Macacus rhesus.—July 1, 1905, 10 A. M. Two thirds culture "Smith" injected. No fluid obtained prior to injection. No symptoms developed, but the canal must have been entered as lumbar puncture July 2, 12 M., yielded a small quantity of turbid fluid containing leucocytes and very few degenerating, intracellular diplococci. No growth was obtained on sheep-serum agar. No symptoms of disease were noted in this animal. July 6, found dead. At the autopsy more clear fluid than is usual escaped from the dura surrounding the cord. The lower segment of the cord was congested. No exudate was visible and the ventricles were not dilated. The pial vessels of the medulla were injected. Smear preparations from the spinal cord and several parts of the brain showed neither diplococci nor polymorphonuclear cells. The sections of the tissue show no inflammation or leucocytic infiltration except superficially in the lumbar region in the posterior columns of the spinal cord at what was probably the point of entrance of the needle.

In contrast to the ease with which the acute inflammations can be excited is the difficulty which attends the production in monkeys of a subacute form of meningitis. The intraspinal injections pro-

duce either an acutely fatal meningitis, or an acute disease from which recovery takes place rapidly. The animals which survive inoculation are usually well of the disease in three or four days. In rare instances, the animal lingers a victim of the disease for a longer period than this to succumb in the end. Monkeys which survive the second day after inoculation tend rather to recover than to die. By following in inoculated monkeys the changes in the cerebro-spinal canal by means of lumbar puncture, the progress of the infection can be traced and the result often predicted. Disappearance of the diplococci early from the canal is a good sign; early emigration of leucocytes is also a good sign; early emigration, active phagocytosis and dissolution of the diplococci, both within and without leucocytes, are very favorable signs. Proceeding in this way, I have been able to keep a small number of monkeys in a state of subinfection with the diplococcus for a period of several days or weeks. The period of successive inoculation was determined by the puncture and by the physical condition of the monkey. A state of resistance tended to develop which necessitated the employment of increasing amounts of cultures to produce visible effects; and in the end the run-down animal succumbed to the dose administered. In these animals the exudates were thicker and firmer and covered the base and the convex surface of the brain; and the ventricles were sometimes widely dilated and contained turbid exudates. The first of these experiments was made with a South American species of which the next protocol gives an account.

Monkey No. 27. South American; Genus Cebus.—October 7, 1905, 11 A. M. Injected suspension of one agar culture "Whitaker," three days old. No symptoms developed. October 9, 11 A. M., l. p.: free flow of turbid fluid, of which 1 c.c. was collected. Injected one "Smith" culture eighteen hours old. 1 P. M., animal crouched in corner; head down and resting in hands; indisposed to move. 5:30 P. M., condition about the same; l. p.: a small quantity of fluid containing leucocytes, but no diplococci obtained. Culture negative. October 12, 3 P. M., l. p. yielded a small amount of turbid fluid containing vacuolated leucocytes and mononuclear cells; no diplococci. One half agar slant culture of "Smith" injected. 9:30 P. M., animal depressed. October 13, animal on perch. October 16, appeared to be well. Suspension of two old "Smith" cultures injected. October 17, no marked effects. L. p. gave a few drops of faintly turbid fluid showing leucocytes with intact nuclei, of which some contain degenerated diplococci. October 18, l. p.: faintly turbid fluid flowed freely from the needle. C. s.: leucocytes and

mononuclear cells; no cocci. A number of vacuolated (or fatty) large mononuclear cells present. Injected one twenty-four hour culture "Smith." No symptoms developed. October 20, 3 P. M., l. p. gave small amount of turbid fluid showing leucocytes but no cocci. Culture negative. Injected one culture "Smith" twenty-four hours old. No symptoms. October 23, l. p.: 0.5 c.c. turbid fluid obtained containing leucocytes and a few red corpuscles. October 28, l. p. gave clear fluid containing mononuclear cells. Injected two "Smith" cultures. October 30, l. p.: a drop of turbid fluid obtained showing leucocytes with fragmented nuclei but no cocci. Injected two "Smith" cultures. October 31, l. p. yielded a few drops of turbid fluid showing many leucocytes, and large mononuclear cells; no definite cocci; culture negative. During this treatment the monkey lost in weight. No further injection was given until November 15, at 12:30 P. M., at which time a heavy suspension of a "Smith" culture, eighteen hours old (representing four agar slant surfaces) was injected. Symptoms rapidly developed; at 2:30, animal sick; l. p. gave with difficulty a few drops of blood-tinted fluid containing many agglutinated diplococci and a small number of leucocytes, of which some had included diplococci. 4 P. M., l. p. gave, with difficulty, little fluid in which diplococci were numerous. October 16, 9 A. M., monkey died. Autopsy. No excess of fluid in the meninges of the spinal cord, and no exudate was visible. Turbid fluid, in excess of the normal, infiltrated the meninges from the medulla to the optic commissure. The anterior and lateral pial surfaces of the hemispheres showed smaller and larger ecchymoses. The ventricles contained small quantities of turbid fluid; they were not dilated. Cultures from different parts of the brain and cord remained sterile. The cover-glass preparations from different levels of the spinal cord, the base and convexity of the brain, choroid plexus and ventricles differed from one another only in minor details. Several striking conditions were shown by them to exist in the membranes. In the first place, no, or almost no, normal cocci remained, but only fragments of swollen and degenerated cocci which were entirely or nearly so within cells. The cells consisted of polymorphonuclear leucocytes which predominated in number and contained the far greater number of cocci; and of much larger cells of wholly different aspect. The latter were several times as large as the leucocytes, and occasional examples were of colossal size (equal to 20 to 40 leucocytes). The nucleus was single, as a rule, and oval or crescentic in form, and vesicular. The protoplasm was pale, and the membrane at the periphery stained deeply. The smaller of these cells contained at times, degenerated cocci; and all may have ingested a variable number of ordinary leucocytes, or mononuclear cells. They were most numerous in the smears prepared from the exudate of the meninges of the medulla. The sections of the central nervous system present quite a different appearance from the other cases described. Although the exudate is thicker than in the previous instances, the chief novelty lies in the great proliferation of the fixed cells of the pia-arachnoid. The multiplication has taken place chiefly along the surface of the brain and at the outer line of the membrane. The new cells are relatively large and vesicular, and their occurrence in double rows gives at times a somewhat glandular appearance to the membrane. Polymorphonuclear leucocytes are numerous and distributed irregularly among the other cells. The degree

of encephalitis is far less than in many of the experiments. The main arterial branches of the brain show a more or less marked infiltration of the intima with leucocytes, and leucocytic invasion of the sub-intimal and inner-muscular layers of the vessels.

This experiment brings out the remarkable capacity which can be developed in the monkey to dispose of cultures of the diplococcus injected into the spinal canal. It is shown by the experiments that the actively bactericidal cells and secretions of the canal are capable of destroying, in a few hours, prodigious numbers of the diplococcus, and that agglutination of the diplococcus can also take place in the canal. Finally, it is shown that the monkey can be killed by an overdose of culture which causes death by poisoning and not by infecting the animals directly; for the lethal effect occurred in this case, although the diplococci were themselves quickly killed. Increased power, therefore, to destroy the diplococcus is not associated necessarily with an equally increased power to resist the toxic effects of the intracellular poison. This fact must have an important bearing upon a specific therapy of diplococcus meningitis.

The experiment is important in respect to the histological alterations present in the meninges. In no previous experiment did the exudate contain so large a number of cells which differed from leucocytes. The large cells described here occur regularly in cases of human meningitis and have been frequently observed. Their occurrence in numbers in this monkey serves to knit even more closely the experimental and the human diseases. Flexner and Barker,[11] and Councilman, Mallory and Wright[12] describe and figure these large cells among the leucocytic infiltration, and the latter investigators have traced their origin to the cells of the connective tissue and the lymph spaces of the pia-arachnoid. The histological conditions present in the membranes are comparable with those observed in cases of meningitis of longer duration in man.

Monkey No. 32. Macacus rhesus. April 19, 1906, at 12 M., given one agar culture "548" in the third generation. 3 P. M., l. p., no fluid obtained. April 20, 12 M., l. p., small quantity of clear fluid containing few leucocytes and free cocci. 6 P. M., animal depressed. April 21, active. April 22, 12 M., injected

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[11] A contribution to our knowledge of Epidemic Cerebro-Spinal Meningitis. *Amer. Jour. of Med. Sciences* 1894, CVII, 155, 259.

[12] *Op. cit.*, p. 102.

one culture; no apparent effect. April 23, 4 P. M., l. p., very little fluid obtained. April 25, 12 M., one agar culture "Gratz." Fluid obtained before injection which produced no visible effect. April 26, one agar culture "Gratz." No symptoms. May 1, 3 P. M., l. p., yielded slow flow of faintly turbid fluid showing leucocytes and larger cells but no cocci. Injected turbid suspension (equal to four agar cultures). 6 P. M., animal sick. Died during the night, May 2. Autopsy: The spinal membranes contained an excess of fluid. On opening the dura mater a gelatinous exudate was seen to occupy the meshes of the pia. A similar exudate covered the base of the brain and surrounded the foramen of Magendie. The convex surface of the brain was greatly altered by a moderately firm and adherent exudate of white color which occupied the pia-arachnoid. It covered the surface of the convolutions and filled the sulci, thus giving a remarkably smooth appearance to the brain (Plate IV, Fig. 6). The ventricles were considerably dilated and contained an excess of turbid fluid. Smear preparations showed the chief accumulation of leucocytes to be at the base of the brain and the lower portion of the spinal cord. The lateral surfaces of the brain showed fewer and more were present in the choroid plexus than in the fluid of the ventricles. Diplococci were present everywhere; they were intracellular and often degenerated. The number varied: they were abundant at the base and less numerous at the convexity of the brain. The mucous membranes of the nose showed, among the columnar epithelium, a number of fragmented leucocytes carrying diplococci indistinguishable from those of the brain (Plate V, Fig. 7). Sections of the spinal cord and brain bear out the findings described. The exudate occurs throughout the membranes covering these structures. It is thicker over the posterior than over the anterior surface of the cord, and while it is abundant over the convolutions, it is especially thick in the sulci. Little or no fibrin occurs. The exudate consists chiefly of polymorphonuclear leucocytes among which there are a large number of moderately large mononuclear cells. The brain substance is remarkably free from infiltration, while the lumbar cord shows invasion, about its posterior surface, to a considerable depth. The dura of the cord at certain levels shows leucocytic infiltration. Diplococci which are very numerous in the exudate, are almost entirely within leucocytes. They have been carried by these cells into the substance of the spinal cord.

A striking feature of the sections is derived from the width of the ventricles. As a rule, these appear as slits in the sections; in this case they are wide cavities. Usually, the ependymal epithelium is regular and relatively high; in this case, it is often depressed or flattened, and a considerable flattening of the choroid plexus, toward the wall of the ventricle, is noticeable. A considerable degree of sub-epithelial cellular proliferation has taken place in the walls of the lateral and fourth ventricles. Leucocytes are moderately abundant in the ventricles.

The dura mater was removed with its attachments to the ethmoid bone, and longitudinal sections prepared. The leucocytes surround the olfactory bulbs in dense masses, and they push along clefts and spaces in the dense membrane, existing now as elongated columns and now in circumscribed, abscess-like masses.

This experiment is valuable in showing that a condition of subacute infection of the meninges can be procured in the monkey by

the use of extraordinary means, and when the condition is present and the thick exudate incidentally covers and occludes the foramen of Magendie, a degree of internal hydrocephalus develops. The experiment enforces a point already made. The histological study of the dura mater at the anterior extremity shows that leucocytes pass into its substance for a considerable distance beyond the proper confines of the cranial cavity. The cells could, indeed, easily carry diplococci with them, in which case an explanation for the occurrence of leucocytes carrying diplococci in the nasal mucosa might be readily found.

The series of experiments described suffices to establish the varieties of effects which are produced upon the central nervous system of lower monkeys by means of intra-spinal injections of cultures of *Diplococcus intracellularis*. Among the histological appearances mentioned, there was one monkey, No. 17, which deserves more particular attention. I described, in this case, a condition of acute endarteritis of the type frequently found in cases of human meningitis. It would appear that this pathological condition occurs in many forms of meningitis as is shown by the investigations of Hektoen,[13] Councilman, Mallory and Wright,[14] and others. The latter authors state, indeed, that it is less commonly found in the epidemic (*Diplococcus intracellularis*) form of meningitis than in other forms (pneumococcus, etc.) of the disease. They figure a slight degree of the condition in a goat in which they produced experimental meningitis by injecting the diplococcus. I have encountered in still another monkey inoculated with the diplococcus very extensive acute endarteritis. The case will be described in full in my article on a serum therapy for experimental meningitis. A spider monkey (*Atelas ater*) was inoculated on December 2, 1905, at 12 o'clock noon, with a slant agar culture of the diplococcus. At 3 P. M. two cubic centimeters of anti-goat (diplococcus) serum were injected into the spinal canal. Death occurred December 3, 11 A. M. The lesion mentioned affects arteries of all sizes in the brain. At the base, over the medulla, rarely does

[13] The Vascular Changes of Tuberculous Lepto-meningitis, especially the Tuberculous Endarteritis, *Jour. Exp. Med.*, 1896, I, 112.

[14] *Op. cit.*, p. 77.

an artery escape. The arteries of the convexity are affected less uniformly, and those of the spinal cord least of all. The systemic arteries do not show the lesion. The subendothelial infiltrations of mononuclear and polynuclear cells may or may not occlude the vessel entirely (Plate V, Fig. 8). Both large and small vessels may be partially or wholly filled up with cells which, as a rule, push the endothelium before them. The endothelium is preserved unless lost from mechanical causes in making the sections. An appearance has been obtained in some vessels of a mushroom-like overlying of the endothelium by the new cells which have accumulated in the adjacent part of the vessel. The muscular coat is usually free from infiltration, as are the veins. This monkey showed an unusual degree of hæmorrhage into the meninges, and definite cortical abscesses, of small size, extending into the brain from the cortical membranes, or developing about the intra-cortical blood-vessels. The number of diplococci in the inflammatory exudate was not large.

The pathological effects and appearances which have been described are not, probably, specific. The experiments are, therefore, valuable, not because they show that *Diplococcus intracellularis* alone of the pyogenic micrococci can produce acute meningitis in monkeys, but because they prove that the diplococcus is capable of setting up in these animals lesions of meningitis which bear a close resemblance to the lesions which the same micro-organism causes naturally in human subjects. The finer the details of correspondence are between the natural and experimental disease, the more valuable do the experimental results become, and the more convincing are they that *Diplococcus intracellularis* is really the cause of epidemic meningitis in man. I have not undertaken to study all the common pyogenic micrococci in the manner of this study of the diplococcus, but I have made, for obvious reasons, a single experiment with *Micrococcus catarrhalis*. The culture employed was kindly given me by Dr. Elser. It fulfilled the usual biological tests for that micro-organism.

Monkey No. 28. South American; Genus Cebus.—October 12, 1905, at 3 P. M., inoculated three agar slant cultures of *M. catarrhalis*. Clear fluid flowed from the needle before the injection. 9 A. M., animal sick; sat on

bottom of the cage. October 13, 9 A. M., condition unchanged. 3 P. M., very sick; head rested on hands. L. p., 9 A. M., yielded a very small quantity of fluid by using syringe. C. s., many leucocytes containing intracellular cocci in moderate numbers; a few cocci extra-cellular. The cocci appeared larger than the diplococcus; some stained feebly. October 16, animal had lost flesh but was recovering. Sat on perch. L. p., 3 P. M., with the syringe a few drops of faintly turbid fluid obtained. It showed small numbers of leucocytes with fragmented nuclei, but no cocci. This animal never fully recovered its flesh, and the hair entirely disappeared from the long tail. It was found dead on February 12, 1906. The autopsy showed the brain and cord to have returned, apparently, to the normal condition. Cover-slips were free of leucocytes and cocci; cultures remained sterile. An adequate cause of death was not found. Nearly all of this species of monkey contain adult filaria in the peritoneal cavity, and filarial embryos in the blood.

. . . .

By means of the experiments recorded in this paper, it has been shown that the lower monkeys can be infected without great difficulty with *Diplococcus intracellularis* and made to reproduce the pathologic conditions present in man in cerebro-spinal meningitis. The experiments establish that the diplococci, when introduced in a low level of the spinal canal, distribute themselves in a few hours through the meninges and excite an acute inflammation, the exudate of which accumulates chiefly in the lower spinal meninges and the meninges of the base of the brain. The uniformity with which the chief exudate is found at the base of the brain and the rarity of its appearance in equally great amounts over the convexity is a fact of importance in the dynamics of the cerebro-spinal fluid since there is relation between the quantity of exudate and the accumulation of the diplococcus. This distribution of the exudate led me to doubt the validity of the reasoning which would ascribe this localization of the inflammation in man to the entrance into the meninges of the infective agent directly through the nasal membrane. This tendency to localization at the base of the brain in monkeys is especially interesting in view of the fact that it is usually only a short time before death that they lie down upon the side of the body. In comparing the experimental lesions in monkeys with the naturally developed lesions in man, note should be taken of the occurrence under both conditions of encephalitis and abscesses, of hæmorrhages, of proliferation of large cells of connective tissue and tissue spaces, of acute endarteritis, of inflammation of the dorsal

root ganglia, of internal hydrocephalus, of relatively small amount of fibrin in the exudates, of fibrinous and other thrombi, and of phagocytosis of diplococci and somatic cells.

The inflammation of the meninges extends in monkeys into the membranes covering the olfactory lobes and along the dura mater into the ethmoid plate and nasal mucosa. The nasal mucous membrane is found, in many instances, to be inflamed and beset with hæmorrhages. Smear preparations from the nasal mucous membrane, from the higher levels especially, have shown me polymorphonuclear leucocytes, often in numbers, carrying diplococci which presented the form, size, staining qualities and degenerations of the diplococci occurring in the same cases in the cerebral and spinal meninges. Thus far *Diplococcus intracellularis* has not been cultivated from the nose of the infected monkeys. I have, however, secured other Gram-negative diplococci with which, it may be stated, the intracellularis could not be confounded.

Although the pathological effects produced in monkeys are comparable with those occurring in the natural disease in man, there is no real correspondence in the relative degree of susceptibility of the two species. The quantity of an active culture required to cause marked symptoms or to bring about death from meningitis in monkeys, is prodigious if compared with the number of diplococci which probably suffice to produce infection in human beings. Moreover, the amount of multiplication of the diplococcus in monkeys, excepting possibly in the focal abscesses, is under the most favorable conditions, small; and I am not disinclined to believe that in many of the experiments no multiplication whatever took place.

The experiments, the details of which have been set down here, besides being an argument for the causative action of *Diplococcus intracellularis* in epidemic meningitis, form, also, the basis of an attempt to influence the progress and termination of the experimental infection in monkeys through the employment of anti-sera prepared from the diplococcus. The results of the experiments with the anti-sera are given in a separate article.

I wish to acknowledge the valuable aid rendered by my former assistant, Dr. H. S. Houghton, in the course of these experiments.

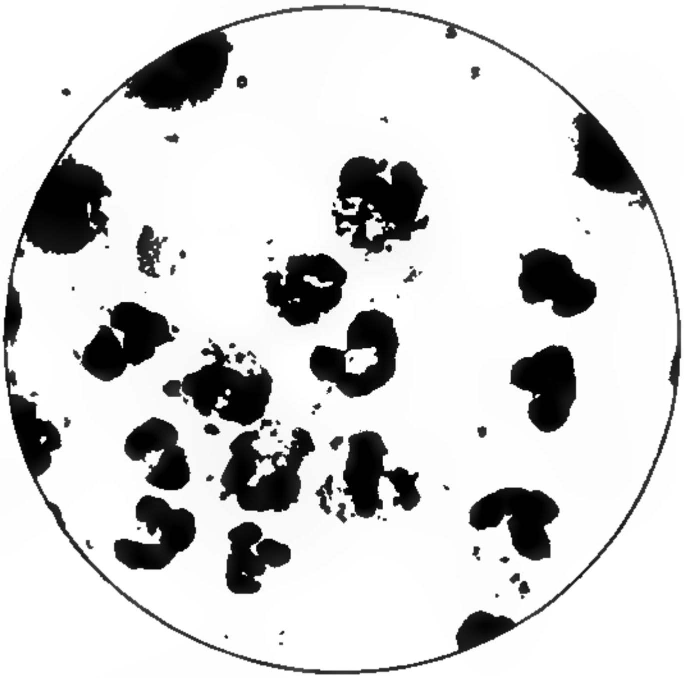

FIG. 1.

FIG. 2.

Fig. 3.

Fig. 4.

Fig. 5.

Fig. 6

Fig. 7.

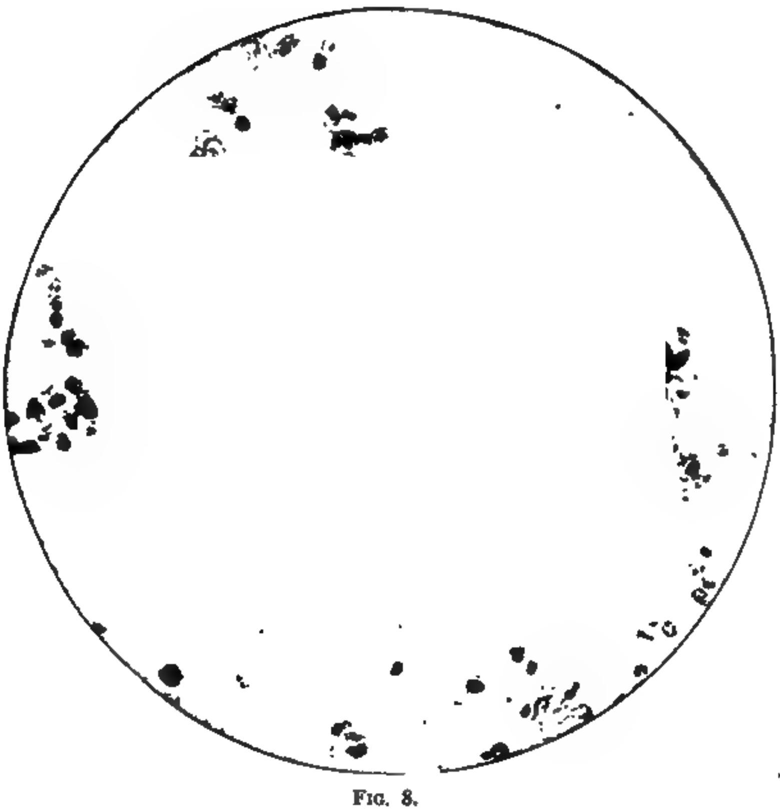

Fig. 8.

DESCRIPTION OF PHOTOGRAPHS.

Plate II.

Fig. 1. Smear preparation from contents of brain abscess, monkey No. 1. Typical intracellular diplococci. Methylene-blue staining. $\frac{1}{12}$ in. objective.

Fig. 2. Low magnification to show the relation of the exudation in the pia-arachnoid of the convex surface of the brain and the sulci, and the extension of the exudation along the cortical vessels into the brain.

Plate III.

Fig. 3. Spinal ganglion showing leucocytic invasion about and between the nerve-cells of the ganglion.

Fig. 4. Low magnification to show the degree of acute encephalitis, and the involvement of intracortical blood-vessels in the infectious processes.

Plate IV.

Fig. 5. Low magnification to show the exudation about and within the spinal nerves.

Fig. 6. Brain, natural size, from a case (Monkey No. 32) of subacute meningitis compared with the brain from a case of very acute experimental meningitis in which the congestion of the pial vessels is very marked.

Plate V.

Fig. 7. Smear preparation from inflamed nasal mucosa showing a leucocyte, *partly* broken, enclosing diplococci, lying among columnar ciliated epithelial cells.

Fig. 8. High magnification of an artery of the base of the brain showing the lesion of acute endarteritis.

I am indebted to the skill and kindness of Dr. Edward Leaming for the *photographs* illustrating this paper.

CONCERNING A SERUM-THERAPY FOR EXPERIMENTAL INFECTION WITH DIPLOCOCCUS INTRACELLULARIS.

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The high mortality of epidemic meningitis and the deplorable deformities caused by it demand that incessant effort be made to discover therapeutic measures which may mitigate the consequences of the disease. The epidemic through which the city of New York has recently passed, and the almost co-incident Silesian epidemic, have been scientifically fruitful in establishing more firmly the belief in the spread of the disease through immediate or mediate contact with the sick, and in tracing the common occurrence of *Diplococcus intracellularis* in the nasal and pharyngeal secretions of the sick, and the exceptional occurrence of the micro-organism in these secretions in the well who have been in contact with the sick. This mode of spread of the disease through directly and indirectly infected persons must come to exercise an important influence on the hygienic measures which will be enforced hereafter to limit the dissemination of the disease.[1]

[1] Goodwin and Sholly: The frequent occurrence of meningococci in the nasal cavities of meningitis patients and of those in direct contact with them. *Journal of Infectious Diseases*, 1906, Supplement No. 2, p. 21.

Flatten: Die übertragbarre Genickstarre im Regierungsbezirk Oppeln, im Jahre 1905 und ihre Bekämpfung. *Klinisches Jahrbuch*, 1906, xv, 211.

Schneider: Idem im Regierungsbezirk Breslau, *ibid.*, p. 300.

Rieger: Idem im Kreise Brieg, *ibid.*, p. 321.

Schmidt: Idem im Regierungsbezirk Leignitz, *ibid.*, p. 341.

Flügge: Die im hygienischen Institut der königl. Universität Breslau während der Genickstarre-epidemie im Jahre 1905 ausgefuhrten Untersuchungen, *ibid.*, p. 353.

v. Lingelsheim: Die bacteriolog. Arbeiten der kgl. hyg. Station zu Beuthen, etc., *ibid.*, p. 373.

Göppert: Zur Kenntnis der Meningitis cerebro-spinalis-Epidemica mit besonderer Berücksichtigung des Kindersalters, *ibid.*, p. 313.

A less certain advance has been made in the therapeutics of epidemic meningitis. The one therapeutic measure growing out of the study of the epidemics in America and Germany which offers any hope is an antiserum for the diplococcus. It is true that the experience of the past is not favorable to the hope of achieving remarkable success by the employment of antibacterial immune sera. All indications point to the pathological effects of the diplococcus as being caused by endotoxic constituents; and thus far, according to many investigators, these endotoxins have failed to yield, by methods of immunization, active antisera which have proved valuable in the treatment of infectious diseases. Opinion is, however, considerably divided on this subject;[2] and in the absence of more certain methods of reaching the desired goal tests of antisera for the diplococcus are certainly justified. These tests can in the preliminary stages be carried out on certain animals, since the course of infection in them with the diplococcus is now fairly well understood.[3]

The main question which would seem to be involved in the search for an active antiserum against meningitis is whether the quantity of antibody which can be produced will suffice to neutralize such a quantity of the poison of the diplococcus as to influence the result of the infection. In fact, the problem may not be so simple, or, indeed, so hopeless, as this proposition indicates. It is, of course, important that neutralization of the poison should if possible be secured, but the effect of the restraint of growth and multiplication of the diplococcus may, at some periods of the disease, be of greater significance than the neutralization of free endotoxin. Fortunately, many agents, some of them quite indifferent, are able to affect the power of multiplication in the body of the diplococcus. It has been shown, indeed, that serum in the fresh state and after

Meyer: Bericht über rhinolog. Beobachtung bei der Genickstarre-epidemie, 1905, *ibid.*, p. 427.

Westenhoeffer: Pathologisch-anatomische Ergebnisse der oberschlesischen Genickstarre-epidemie von 1905, *ibid.*, p. 447.

Jehle: Entstehung der Genickstarre-epidemie, Wien. klin. Woch., 1905, xix, 25.

[2] Besredka, *Annales l'Institut Pasteur,* 1906, xx, 4.

[3] Attention is directed to the two previous papers of this series published in this number of the Journal.

heating to 60° C., preserves the power to destroy in test-tubes large numbers of the diplococcus, and sterile fluid inflammatory exudates possess this power in even greater degree. An antiserum, therefore, even though it contain relatively small amounts of antibodies, as indicated by neutralization experiments, may be effective beyond this calculated value by restraining the multiplication of the diplococci, possibly by reducing outright their number, and by supporting the power of resistance normally at the disposal of the body.

The conditions are made theoretically less discouraging, perhaps, because the main pathological lesions are limited to the cavity of the cerebro-spinal axis. They can, therefore, be brought directly under the influence of the antisera by injecting the latter into the spinal canal. A large advantage is gained by this circumstance. It is, on the other hand, discouraging to reflect that in monkeys infected with the diplococcus, severe cortical lesions already exist at the end of ten or twelve hours. The question arises whether these deeper lesions tend to appear as early in the human infections. In respect to this question it should be stated that observation is against the occurrence of any such development of the diplococcus in the early stages of the human disease as is represented by the prodigious number of diplococci required to be injected into monkeys to produce the rapidly lethal effect with which the cortical lesions are associated. It is worthy of note that the more slowly developed lesions in the monkey remain more superficial, agreeing in this respect with the more common lesions present in fatal cases of the human infection. Hence, some encouragement may be taken from the power of the antiserum to influence favorably the course of meningitis in the monkey, although it has been injected as late as six hours after the inoculation.

If we are at all permitted to apply test-tube experiments to what may happen in the body, it would not be remarkable if the normal serum of animals, and perhaps of human beings, proved to be beneficial to a degree when brought into direct relation with the focus of development of the diplococcus. At first sight, judging from test-tube experiments, it would appear as if the exudate, called out by the inflammation, should suffice to destroy the diplococci; this manifestly does not happen in many cases. Indeed, it

is found that incubation outside the body will even increase the number of diplococci in the inflammatory fluid withdrawn from the spinal canal. It is safe to assume, therefore, that the exudate withdrawn has been exhausted of its power to destroy the diplococcus. It is quite possible that the introduction of fresh serum, of the same species of animal, may be helpful by bringing quickly into contact with the diplococci a quantity of actively destructive serum. The results of some of my experiments show that normal serum reduces appreciably the toxic effect of given doses of the diplococcus.

In experiments upon the monkey there is a definite low limit, beyond which it is not safe to go, for injection of fluids into the spinal canal. The species which I studied contain a small amount only of free spinal fluid. If one attempts to inject several cubic centimeters of fluid, symptoms of pressure may develop. In this respect the monkey is far less satisfactory to treat by intraspinal injections than are human beings.

I am far from having any conviction that cerebro-spinal meningitis in man can be influenced favorably by injections of immune sera into the spinal canal, or elsewhere in the body. The experiments to be described merely show that guinea-pigs and monkeys, in which the conditions of infection can be controlled, can be saved from the otherwise fatal effects of the diplococcus by the use of antisera, and to a less extent by the use of normal sera and other fluids. A preliminary note on this subject has already been made.[4] The protocols show that the experiments on immunity were begun during the spring of 1905. While the work was in progress two papers on the same subject appeared in Germany.[5] The use of monkeys for testing the antisera by direct injections into the infected and inflamed cerebro-spinal canal has not been made by the other investigators whose experimental studies were confined to guinea-pigs. Jochmann injected an antiserum prepared in the horse into the spinal canal of several human subjects of epidemic meningitis. The number of cases was too few to permit any conclusion of the value of the injections; but they showed that the injection of

[4] *Jour. of Amer. Med. Assoc.*, 1906, xlvii, 560.

[5] Kolle and Wassermann, *Deutsch. Med. Wochenschrift*, 1906, xxxii, 16. Jochmann, *ibid.*, p. 20.

horse's serum into the inflamed canal is not attended with special danger.

My first experiments on guinea-pigs were made with goat's sera. A female goat had been injected twice with cultures from several sources (12) of the diplococcus within a period of two weeks. The injections were made subcutaneously and gave rise to tumefaction which soon disappeared. After the second injection the goat aborted. The first bleeding was made two weeks after the second injection. As the table shows the serum at this time had little or no immunizing power. The experiment was designed to test the effect of an injection (1) previous to the injection of the diplococci, and (2) after the inoculation of the diplococci. The serum in the first instance was injected at 5 P. M. the day before, and in the second instance two hours after the inoculation with the diplococcus. All the injections, except bouillon in one pig, were intraperitoneal. The emulsion of the diplococcus was injected at 11 A. M., November 29, 1905.

Series No.	Weight in Grams	Protective Substance Injected.	Result.
144	189	"Immune" serum 0 03 c.c. 5 P.M. Nov. 28	Died 9 A.M. Nov. 30.
145	189	" " 0.04 c.c. " " "	Died 8 A.M. Dec. 2.
146	182	" " 0.05 c.c. " " "	Survived.
147	197	"Normal" " 0.05 c.c. " " "	Survived. Lost weight.
148	197	" " 0.10 c.c. " " "	" " "
149	220	Bouillon intraper. 1.0 c.c. " " "	" Weight 12/11 182 grams.
150	199	" subcut. 1.0 c.c. " " "	Died morning, Dec. 3.
151	190	"Immune" serum 1.0 c.c. 1 P.M. Nov. 29	Survived. Lost weight.
152	185	" " 2.0 c.c. " " "	" " "
153	192	"Normal" " 1.0 c.c. " " "	Died 5 P.M. Nov. 30.
154	190	" " 2.0 c.c. " " "	Died in night, Nov. 29.
155	190	Nothing: control	Died morning, Dec. 1.
156	210	Idem.	Died in night, Nov. 29.

The preceding experiment is of interest in showing the irregular action of the serum injections, and especially, as bringing out the fact that such an indifferent substance as bouillon can, if it is injected in advance of the diplococcus, impart power of successful resistance to the guinea-pig. It developed subsequently that the bouillon need not be injected into the peritoneal cavity to achieve this effect; one cubic centimeter injected subcutaneously in pigs of 225 to 250 gram weight, the day before inoculation, frequently

saves the animals. Using the goat serum of low protective value, the fact was determined that the protecting power of the serum has a definite limit (about 0.05 c.c. for pigs of 250 grams weight) under the most favorable conditions, namely, intraperitoneal injection the day before the infection. By using larger quantities (up to 1.0 c.c.) of serum simultaneously with the injection of the diplococcus the pigs can also be saved; subcutaneous injections of the serum (1.0 c.c.) in advance are effective. The smallest number of successful results is obtained in pigs in which the serum injections follow, after one or more hours, the infection. Several experiments were carried out in order to determine the fate of the diplococcus in the peritoneal cavity in the "protected" and "unprotected" animals. The plan was to withdraw fluid, after a suitable interval, by means of capillary tubes. The results were not wholly uniform, but in the main showed more rapid disappearance of the

Guinea-pigs weighing 230 to 260 grams, received 0.1 c.c. "immune" goat's serum intraperitoneally and subcutaneously, 1.0 c.c. bouillon intraperitoneally and subcutaneously, followed the next day by an emulsion of the diplococcus injected into the peritoneal cavity.

Series No.	How Injected.	Examination During Life.	Autopsy Findings in Peritoneum.
119	Goat's serum intraperitoneal; emulsion cocci at 11:30 A.M.	4 P.M. Many leucocytes containing a few diplococci; no extracellular diplococci.	Survived.
120	Goat's serum subcutaneous; emulsion cocci at 11:30 A.M.	4 P.M. Enormous number of diplococci; very few leucocytes overlaid with diplococci.	Died during the night. 2 c.c. fluid exudate; more pus present than usual; many leucocytes including diplococci.
121	Bouillon intraperitoneal; emulsion cocci at 11:30 A.M.	4 P.M. Diplococci more numerous than in 120; no leucocytes.	Died during the night. 3 c.c. fluid exudate; no leucocytes; many diplococci.
122	Bouillon subcutaneous; emulsion cocci at 11:30 A.M.	4 P.M. Many leucocytes; some intracellular diplococci; very few extracellular ones.	Survived.
125	Control; emulsion cocci at 11:30 A.M.	4 P.M. Enormous number of diplococci; almost no leucocytes.	Died during the night. Usual p. m. appearances; very large number of diplococci free; almost no leucocytes. The omentum contains some leucocytes including cocci.

diplococcus from the peritoneum of the treated as compared with the untreated pigs. Exudation of cells was more abundant in the former animals.

The injections of the goat were carried on subsequently, during and after lactation, until the end of February, 1906. Blood was withdrawn from time to time and the serum tested for its protective and therapeutic value. The results were never uniform, but the general indication was that the protective properties were increased measurably. The animal fell ill on March 15, 1906, and was bled to death. The serum obtained from this bleeding was used for many subsequent experiments. It was found by simultaneous injection to protect small pigs (190 to 200 grams) against a twelve hour fatal dose of the diplococcus in quantities varying from 0.5 to 0.01 c.c., but not regularly. On the whole, as the next experiment given in detail will tend to show, the serum had acquired protective and therapeutic properties.

An emulsion of the diplococcus was prepared of which 0.5 c.c. caused death in the control pigs in about eighteen hours. Three series of pigs were tested; the first received the serum and emulsion simultaneously, the second the serum two hours, and the third the serum four hours after the emulsion. One tenth c.c. of serum was injected in each pig. The emulsion was given intraperitoneally.

Series No.	Serum Injected.	Result.
102	Intraperitoneal; simultaneously.	Survived.
103	Subcutaneous; "	Died in 4 hours.
105	Subcutaneous; after 2 hours.	" 16 "
106	Intraperitoneal; " 2 "	Survived.
107	Subcutaneous; " 4 "	Died in 56 hours.
108	Intraperitoneal; " 4 "	Survived.

I shall supplement this table by another in which the goat serum is compared with an immune serum made in monkeys (vide infra). This experiment has, in this place, a two-fold value in showing that certain of the series of tests proceed in a regular manner, and the two immune sera have about equal protective value for guinea-pigs. The emulsions of the diplococcus killed six control pigs in from nine to fifteen hours. The injections were simultaneous.

The next table given shows that the sera of the goat and the monkey were both capable of protecting guinea-pigs from fatal doses of the diplococcus by simultaneous injection, and the previous

Series No.	Weight in Grams.	Serum Injected.	Result.
106	190	0.1 c.c. goat, interperitoneal.	Survived.
107	188	Idem.	"
108	180	0.1 c.c. monkey, intraperitoneal.	"
109	185	Idem.	"
110	185	0.1 c.c. goat, subcutaneous.	"
112	175	Idem.	Died in 12 hours.
114	195	0.1 c.c. monkey, subcutaneous.	Survived.
117	178	Idem.	"
175	210	0.1 c.c. goat serum, intraperitoneal.	"
178	205	0.2 c.c. " " "	"
181	170	0.5 c.c. " " "	"
188	185	1.0 c.c. " " "	"
182	205	0.1 c.c. monkey serum, intraperitoneal.	"
183	182	0.2 c.c. " " "	"
186	175	0.5 c.c. " " "	"
187	172	1.0 c.c. " " "	Died in 30 hours.

one indicates that the infected pigs can with less uniformity be rescued at the expiration of two to four hours after inoculation. The striking results of the last table are, however, relative merely, since it is found that certain normal goat sera possess a power that it almost equal to that of the immune serum in protecting by simultaneous inoculation. On reweighing, four days later, the surviving pigs of the last tabulation a marked loss of weight was found to have taken place in those which received the sera subcutaneously and not in those receiving it into the peritoneum.

It would be extremely hazardous to express, on the basis of my experimental results, the relative value, as protective agents against the diplococcal infection in guinea-pigs, of normal and immune goat sera. I was restricted to the use of a female goat for the purpose of immunization, which was, unfortunately, during a part of the process, lactating. This fact could account for the low relative value of the serum. I did not, however, find the injection of normal goat serum following the inoculation of the diplococcus, so effective as the injection of the immune serum in this way. But here again, I was, I should state, limited in this study to three specimens of normal serum. On the whole, the immune serum saved more guinea-pigs than the normal serum. I am not, however, convinced that in the instances of preceding or simultaneous injection of the serum, in respect to the inoculation, any very great stress can be laid upon the inherent antitoxic, or even bactericidal power of the specific serum, since, in the former case, the exudate

caused to be poured in the peritoneal cavity has itself, in vitro, the power of suppressing large numbers of diplococci, and, in the latter, the sera possess this power only in less degree than the exudate. If, however, it can be shown that the immune sera are more effective as therapeutic agents than normal sera, and by simultaneous and preceding injections, also, then it will have to be conceded that they contain some useful elements absent from the normal serum or present in it in less amount.

In the course of these experiments the effort was made to produce an immune serum in the horse. For this purpose, recent cultures were injected by Dr. Jobling, first subcutaneously and later intravenously. The doses had to be carefully chosen on account of the high sensibility of the horse to the diplococcus. The subcutaneous injections produced local swellings which frequently softened and discharged externally. After a period, intravenous injections of autolysates were begun; but the alarming symptoms which followed almost immediately the injection of a few cubic centimeters of this fluid caused a return to the use of cultures and autolysates by the subcutaneous method. After about five months of intermittent injection the serum was collected and compared for protective value on small guinea-pigs. I employed as controls several samples of normal horse serum kindly supplied by Dr. Park of the Board of Health. I shall give a few typical experiments from which it would appear that on the whole the serum of the treated horse has greater protective value than the serum of normal horses.

Two strains of the diplococcus of moderate virulence were employed in the tests. The suspensions were made in the morning, control pigs being inoculated with them immediately. About four hours later, the rectal temperatures indicated the probably fatal doses. It was subsequently found that 0.1 c.c. of the suspensions caused death in less than twelve hours and represented, for susceptible pigs of about two hundred grams, two or three fatal doses. The inoculation of the pigs for the experiments on immunity was made in the afternoon of the same day. This procedure is rendered necessary by the rapid deterioration of the suspensions in salt solution and Ringer's fluid which sometimes takes place at

the refrigerator temperature. The deterioration is associated with loss of viability and not with striking morphological changes in the diplococci, from which fact I have been led to believe that multiplication for a period of the inoculated diplococci may be a necessary condition of the lethal dose.

Three control guinea-pigs were injected at 10 A. M. with 0.1, 0.2, and 0.3 c.c. of a suspension of "Mt. Sinai culture" of the diplococcus. The pig receiving the largest dose died at 4.30 P. M., at which time the temperatures of the other pigs were 34.6 and 34.8° C., respectively. A series of guinea-pigs which had been injected into the peritoneum at 4 o'clock the previous day with normal and immune horse serum, were now inoculated into the abdominal cavity with the suspension of the diplococcus. The two remaining control pigs died at 11.30 P. M. the same day. All the pigs weighed between 165 and 190 grams.

NORMAL HORSE SERUM.

Series Number	Quantity of Serum Injected 24 Hours Previously.	Quantity of Suspension Injected.	Result.
68	0.01 c.c.	0.2 c.c.	Died midnight.
69	0.02 "	" "	Died 3 A.M.
72	0.05 "	" "	Died midnight.
75	0.07 "	" "	Survived.
78	0.10 "	" "	Died midnight.
82	0.30 "	" "	Died 1 A.M.

IMMUNE HORSE SERUM.

47	0.01 c.c.	0.2 c.c.	Died 3 A.M.
50	0.02 "	" "	Survived.
58	0.05 "	" "	Died 9 A.M.
59	0.07 "	" "	Survived.
61	0.10 "	" "	Died 3 A.M.
66	0.30 "	" "	Survived.

This result, while it is not remarkably good, does, on the whole indicate that the immune serum possesses greater power of protection than the normal serum. The condition of the experiment was fairly severe, since the dose of suspension represented about five fatal doses; but what should be especially noted is the varying sensitiveness of the inoculated pigs owing to which the tests give quite irregular results.

The next tabulation, which would seem to bring out the same fact, gives the result in a series of simultaneous inoculations. The

mixtures of suspension and serum were permitted to stand at the warm room temperature for half an hour before injection.[6]

Series Number.	Serum Injected.	Suspension Injected.	Result.
NORMAL SERUM NO. 1.			
51	0.01 c.c.	0.1 c.c.	Died after 8 hours.
54	0.02 "	0.2 "	" " " "
46	0.10 "	0.5 "	Survived.
NORMAL SERUM NO. 2.			
49	0.01 c.c.	0.1 c.c.	Survived.
43	0.02 "	0.2 "	"
38	0.10 "	0.5 "	Died after 8 hours.
IMMUNE SERUM.			
41	0.01 c.c.	0.1 c.c.	Survived.
37	0.01 "	0.1 "	"
34	0.02 "	0.2 "	"
44	0.02 "	0.2 "	"
42	0.10 "	0.5 "	"
39	0.10 "	0.5 "	"

Two other kinds of antisera were prepared and tested upon guinea-pigs. The first was made by injecting large rabbits with the peritoneal exudate of guinea-pigs which succumbed to intraperitoneal inoculation with the diplococcus. The exudates were toluolized, freed from cells, the toluol was removed by evaporation at a low temperature, and injected into rabbits. The antisera obtained from the rabbit exercised a degree of protection which can be expressed as follows: simultaneous injections of the diplococcus and the antiserum into the peritoneal cavity tend to give protection; separate simultaneous injections, diplococcus into the peritoneum and serum under the skin, are effective in proportion to the quantity injected. Small doses of the serum (0.1 c.c.) do not protect, but they delay the lethal effect; larger doses (0.5 c.c.) prevent the lethal effect. Dosage of rabbit serum proved to be important; too little failed to protect, and too much (0.5 c.c. into the peritoneal cavity) prejudices the result by reason of its own toxicity. The pigs which received the culture and this dose of serum died soon after the controls. The second was an homologous serum

[6] It is my intention to make a later report, when the immunization shall have gone farther, upon the effects of the serum of the immunized horse as regards its power of protection for guinea-pigs and for monkeys.

yielded by large guinea-pigs which were injected at intervals for several months with cultures of the diplococcus, the peritoneal exudate from other guinea-pigs, and the autolysate, which proved not to have greater protective value than normal guinea-pig's serum.

If the result of these attempts to produce an antiserum for *Diplococcus intracellularis,* which should be effective in the experimental infection with the diplococcus in the guinea-pig are reviewed, they cannot be held to be particularly promising. Under the severe conditions of the experiments, the most that can be said is that various agents—bouillon, normal sera, immune sera—can at times affect favorably the course of the experimental infection; the lead in respect to this influence being taken rather by the immune sera. It is to be recalled that in small guinea-pigs the experimental infection is rapidly fatal; that the prostration of the pigs develops very quickly, and the animals are often moribund in six to eight hours after inoculation. To influence very favorably and systematically a pathological process which progresses as rapidly as this, would be, perhaps, an achievement. As the experiments show, this can be done, although not wholly in this degree, by appropriate dosage of certain antisera.

The next experiments to be reported relate to monkeys inoculated with the diplococcus and treated with anti-diplococcus serum made in the monkey. Two large monkeys (*Macacus nemestrinus*) were immunized, for the production of an homologous serum, by injecting them subcutaneously with exudates from the peritoneal cavity of guinea-pigs succumbing to diplococcus infection, and with emulsions of the diplococcus. The injections were made at intervals for a period of nine months, after which the animals were bled to death, and the sera tested. Before giving the protocols of these experiments, the chief facts of a much earlier experiment to influence the course of meningitis in a monkey by means of the antiserum of the goat should be given.

December 2, 1905, each of two spider monkeys (*Atales ater*) was inoculated with one agar slant culture of the diplococcus. Fluid flowed from the needles before injection. 3 P. M., control sick; mate to this perhaps not quite so sick. Into the spinal canal of the latter 2 c.c. of goat's immune serum were injected. The immediate effect was alarming: animal relaxed, heart's action tumultuous,

respiration sighing. The symptoms passed off in 10 to 15 minutes. L. p. before injection of serum showed in each many diplococci; no leucocytes. 9 P. M., control in stupor from which he could be aroused; easily handled. Responded to introduction of needle for l. p., no fluid obtained; point of needle carried a small amount of exudate which showed many leucocytes and a small number of intracellular cocci. Rectal temperature 40° C. Serum treated, less stupid than control; could be handled alone. No fluid obtained by l. p. The small amount of exudate on needle showed leucocytes some of which contained diplococci. Rectal temperature 36° C. December 3, 6 A. M., both still very sick; 8 A. M., serum treated animal very much depressed; 10 A. M., serum treated animal dead; control brighter. The latter animal finally recovered. Autopsy on serum treated monkey. The spinal canal showed hæmorrhagic imbibition of the membranes of the lower third of the cord. The pia-arachnoid of the cord was infiltrated with gelatinous-œdematous exudate. Small hæmorrhages beset the pia-arachnoid of the cortex, and the meninges of the brain were infiltrated with an exudate similar to that of the spinal cord. The ventricles contained turbid fluid in small amount. Microscopical examination of sections of the brain and cord show the exudate to be moderate in amount, and to be thicker over the convexity than over the base of the brain. The hæmorrhages in the membranes of the spinal cord are large, and of the brain small. The most striking lesion is an acute endarteritis which effects all sized branches of the arteries of the brain and cord. Very few diplococci can be found.

The symptoms in this animal and the lesions found at autopsy were taken to indicate that goat serum cannot be injected with impunity into the inflamed spinal canal of monkeys.

Preliminary to the tests of the antisera prepared in the two monkeys, the lethal dose of the diplococcus had to be established. A recently isolated culture (Mt. Sinai 596), which had proven virulent for guinea-pigs, was chosen. Two control monkeys were inoculated (1) with 0.5 c.c., (2) with 1.0 c.c. of a suspension of the culture. Brief histories follow:

Control No. 1. *Macacus rhesus.* June 27, 1906, 11 A. M., given 0.5 c.c. suspension. 6 P. M., very sick; June 28, 9 A. M., brighter; 12 M., l. p. small amount of thin fluid obtained; animal very weak. June 29, 8 A. M., moribund; chloroformed at 1 P. M. Autopsy: The membranes of the cord and brain were pale; little visible exudate. C. s. show leucocytes in small numbers over cord and brain; and a few intracellular diplococci. Sections of the tissues confirm these findings; the inflammatory exudate is small in quantity; no marked lesions of the nervous tissue itself are to be seen.

Control No. 2. *Macacus rhesus.* June 28, 12 M., 1.0 c.c. of same emulsion injected (in refrigerator over night). 6 P. M., monkey sick; 9 P. M., very sick; June 29, 9 A. M., died. Autopsy: The meninges were injected and contained small hæmorrhages. The fluid in the pia of the cord was increased, and turbid. The meninges of the brain and the ventricles contained similar fluid in excess; the basal meninges the largest quantity. C. s. show leucocytes and

diplococci throughout the membranes. The spinal membranes contain the largest number of free diplococci. Cultures positive. Sections of the tissues show the lesions of an acute inflammation of the meninges and of the superficial portion of the cortex of the brain.

In making the serum tests, the larger dose of emulsion was always employed. Brief protocols of the experiments follow:

Experiment 1, July 3, 1906: Medium sized *Macacus rhesus* given at 11 A. M. 1.0 c.c. emulsion of diplococcus "596" together with 1.0 c.c. of monkey antiserum No. 1. Fluid flowed from the needle before injection. July 4, 9 A. M., monkey appeared normal; active; on perch. L. p. yielded several drops of faintly turbid fluid, which showed on cover-slips free cocci, and leucocytes, some of which contained diplococci in moderate numbers staining feebly. Cultures from the fluid negative. July 5, 11 A. M., monkey apparently well. Lumbar puncture gave a small amount of clear fluid, which contained neither leucocytes nor cocci. Cultures negative. January 10, 1907, the monkey remained well.

Experiment 2, July 5, 1906: Medium-sized *Macacus rhesus* given 1.0 c.c. suspension of culture "596" at 11:45 A. M. Fluid flowed from needle before injection. At 2 P. M., the monkey was sick. L. p. gave a small quantity of rather thick, opaque fluid. One cubic centimeter of immune serum (monkey No. 2) injected rapidly. Immediately at conclusion of injection pressure symptoms of an alarming character developed. The animal was prostrated for two hours, after which it slowly got better. At 6 P. M., it responded to disturbance. At 8 A. M., next day, the monkey was up and appeared well. It remained so subsequently (January 10, 1907). The cover-slips from the lumbar puncture showed many polymorphonuclear leucocytes and a small number of lymphocytes, many extracellular and a few intracellular diplococci.

Before the next experiment with the serum was made, the culture "596" was again tested for virulence.

A medium-sized *Macacus rhesus* was given 1.0 c.c. emulsion at 10:30 A. M., July 11. 10 P. M., animal sick; on bottom of cage, July 12, 9 A. M., l. p., small quantity of a thin white exudate obtained. C. s. many leucocytes and few diplococci. Died 12:30 P. M. Lived about thirty-eight hours. The lesions found at autopsy were characteristic; exudate existed over the cord and brain, the base of the latter being chiefly affected. The cortical vessels were injected. C. s. showed cocci in the inflammatory exudate of the cord and base of the brain, and fewer in the exudate of the convex meninges and the ventricles. Sections of the brain and cord show marked inflammatory lesions of the usual character. Diplococci are abundant in the exudate.

Experiment 3, July 13, 1906: Moderately large *Macacus rhesus* given at 11:15 A. M. 1.0 c.c. emulsion of Coccus "596." Although no fluid was obtained, the canal was certainly entered; 4 P. M., animal sick; 5 P. M., depressed, but still sat up. L. p. gave a small quantity of turbid fluid, but during the operation the animal collapsed; 1.0 c.c. of immune serum from monkey No. 2 injected slowly. The monkey was watched until 10 P. M.; no progress of the disease. July 14, 10 A. M., animal appeared well. No future symptoms developed up to January 10, 1907. The fluid obtained by l. p. showed many leucocytes with an occasional intracellular coccus, and extracellular cocci.

Experiment 4, July 27, 1906: At 8:45 A. M., a medium-sized *Macacus rhesus* was given one full agar slant culture "610," 18 hours old, suspended in salt solution, into spinal canal Fluid obtained before injection. At 11 A. M., 5 c.c. of antiserum from monkey No. 2, were injected into the skin of the thigh. Before the serum injection the animal was sick; it lay half down on the bottom of the cage. No immediate effect followed from the injection, but the symptoms did not progress. The next day the animal appeared well. L. p. was unsuccessful after the injection until the next day, at which time clear fluid, containing neither cocci nor leucocytes, was obtained. The control for this monkey was a much smaller and weaker monkey of the same species, which succumbed in eight hours.

I do not regard this experiment as entirely free from doubt, but as I was unable to obtain more monkeys at this time, the experiment could not be repeated then.

The series of experiments was up to this point successful, and they indicated that an antiserum to the diplococcus could prevent the development of severe symptoms from following the injection of the cultures of the diplococcus into the spinal canal, and cause arrest of the symptoms which had already set in. This series of tests is, of course, incomplete without corresponding experiments with normal serum with which they may be compared. The latter will follow. But before citing them I wish to record a failure under conditions which, in view of the foregoing results, was wholly unexpected.

Experiment 5, July 20, 1906: Medium-sized *Macacus rhesus* given at 12 M. 1.0 c.c. of emulsion of Coccus "596"; 3 P. M. 1.0 c.c. of antiserum injected intraspinally. This monkey was sick when given the serum and the symptoms progressed fairly rapidly. At 11 P. M. the animal was much prostrated and sat in the cage with head depressed. It died about 7 A. M., July 21. The autopsy showed a very unusual amount of exudation in the membranes of the cord. Cover-slip preparations showed a purulent exudate with large numbers of diplococci, all within polymorphonuclear leucocytes. The exudate in the meninges of the brain and cord showed the diplococci in the same condition of complete phagocytosis, although the number of leucocytes and diplococci was smaller. The fluid withdrawn by lumbar puncture before the serum injection contained large numbers of diplococci and very few leucocytes; only an occasional leucocyte contained diplococci. Cultures made at the autopsy from the meninges of the cord, medulla and cortex, and from the lateral ventricle were positive; those from the heart and bone marrow of the femur were negative. Examination of sections of the brain and spinal cord bear out the macroscopic appearances. The exudate is remarkably thick over the entire nervous system, and is composed exclusively, or nearly so, of leucocytes. The lateral ventricle is shown to have been dilated and to contain pus cells. The brain tissue has escaped invasion with leucocytes, and the intracortical blood vessels are free from thrombi and do not show the perivascular infiltration with pus cells which is commonly present. The spinal cord at the level of the injection shows a superficial invasion with leuco-

cytes, but the higher levels do not show this condition. The dura is, in the former locality, infiltrated with pus cells.

The control for this experiment was a smaller *rhesus* monkey. The injection was made July 19, at 2 P. M., and as symptoms had failed to develop from the small dose given, a second injection of one third culture was made at 6 P. M. At 9 P. M., animal sick; lying down, but when disturbed rose and looked distrait. July 20, 7:30 A. M., lay on bottom of cage, but on being disturbed rose and displayed marked nystagmus; resumed recumbent position. Depression increased during the morning; 12 M., died. The autopsy showed a general thin exudate in the meninges, marked chiefly over the medulla. Cover-slips showed a remarkably large number of cocci which, while chiefly within leucocytes, were abundant outside. In no other experiment was so large a number of microorganisms seen. A suspicious circumstance was found in the appearance of short chain-like groups of cocci, 4 to 6 members long. The cocci were Gram-negative and in size like the diplococcus. Attempt at cultivation failed.

In summarizing these experiments, it may be said that by the employment of an homologous anti-diplococcus serum several monkeys were saved from death due to experimental infection with *Diplococcus intracellularis*. The conditions of the experiments were such that the inoculated monkeys could, by simultaneous injection of serum and culture be prevented from developing severe symptoms, although the diplococci persisted for a period in the spinal canal, and by separate injection of the culture, and six hours later of the serum, the already severely ill monkey could, apparently, be saved from certain death. The experiment in which the serum was used successfully by subcutaneous injection cannot be interpreted without suitable repetitions.

The tests with normal monkey serum to serve as controls for the above experiments were made so as to bring out two sets of facts. In the first place, the value of simultaneous injection of normal monkey serum and a quantity of culture which would cause death in the control animal within twenty-four hours, was studied. And in the next place, the value of the normal serum was studied in monkeys in which the dose of the culture was on the border line —that is, of such a size that certain monkeys survived and others succumbed after a greater period than twenty-four hours. As regards the second series of tests, it may be said that it appeared as if the injection of a mixture of the normal serum and the culture led, in certain cases, to the survival of the monkey after a period of illness which was sometimes severe. The first symptoms ap-

peared very soon—within one or two hours—and grew in intensity for five or six hours, after which they receded. I am, therefore, inclined to attribute to normal serum employed in this way, a certain definite protective value.

The results in the first class of experiments were different. I found that the normal serum not only failed to save the inoculated monkeys, but the injection of the mixture of culture and serum might even hasten the fatal outcome. I wish to speak with some reservations on this point, for I was greatly hampered in this entire series of tests by great difficulty in obtaining at the time a suitable number of monkeys for the experiments. The study must, indeed, be carried much further before a final answer can be given as to the availability of a serum therapy for this experimental diplococcal infection in monkeys. There follow two brief protocols relating to the use of normal serum with a certainly fatal dose of the diplococcus. Two animals of the same species—*Macacus rhesus*—of equal size were employed.

Control Monkey. At 12 o'clock noon, one cubic centimeter of a suspension of the diplococcus fatal to guinea-pigs was injected into the spinal canal. Fluid flowed from the needle before injection. At 5 P. M., the monkey was sick; 9 P. M., lay on bottom of cage, but could be roused. Next morning comatose; died at 2 P. M. Survived the inoculation 26 hours. The lesions found at autopsy were characteristic. Cover-slips showed that the diplococci had to a large extent disappeared. The cultures were negative.

Serumized Monkey. At 12 o'clock noon, one cubic centimeter of the same emulsion used in the previous experiment (in refrigerator over night) mixed with one cubic centimeter of normal monkey serum and placed at 37° C. for half an hour, was injected into the spinal canal. Fluid was obtained before the injection. No immediate effects were noted following the injection. The monkey was already sick at 3:30 P. M., the symptoms increasing with great rapidity. Death took place at 8 A. M. the next morning. Survived 22 hours. The autopsy showed vivid injection of the meningeal vessels, and many small hæmorrhages over the cortical convolutions. Cover-slips showed a rich emigration of leucocytes and almost total disappearance of the diplococci. The cultures remained sterile.

. . . .

I have no desire to attempt to apply, at this time, the results given here of the experiments with the various sera on guinea-pigs and monkeys to human beings the subjects of cerebro-spinal meningitis. The experimental results with the antisera were not sufficiently constant and striking to make this mode of treatment of human cases of cerebro-spinal meningitis of very hopeful au-

gury. On the other hand, it is not improbable that more active antisera, using appropriate means of immunization, may be produced. Possibly, such antisera may prove of value in the treatment by direct spinal inoculation, possibly even by intravenous or subcutaneous injection, in this hopeless disease. The evident disadvantages to which the human patient must always be subject, as compared with the animals used for experiments, arise from the difficulty often encountered of estimating exactly the duration of the disease, and applying the remedy at its most favorable stage. On the other hand, the exceptional cases only in man run so rapid a course, attended with such profound symptoms of intoxication, as are regularly seen in the inoculation disease in animals. The slower and more measured progression of the infection in human beings may, indeed, be a favorable circumstance, provided the treatment can be applied in the early stages and before too severe structural changes have taken place in the nervous system. The fact that normal serum exercises a certain degree of protection might possibly be taken advantage of in cases of human infection. It would, of course, be practicable to obtain normal human serum for such injections. This subject is one which, in view of the gravity of cerebro-spinal meningitis in man and the absence of any efficient therapeutic measure against it, would seem to deserve consideration.

A CONTRIBUTION TO THE BACTERIOLOGY OF RHEUMATIC FEVER.

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PLATE VI.

Since Wasserman in 1899, experimenting with a diplococcus which he had isolated from a case of chorea, produced what he regarded as typical rheumatic fever in a series of eighty rabbits, much has been written for any against his view.

I do not propose in this paper to, in any way, review the position or to criticize the work of other observers. The bacterial origin of rheumatic fever seems to need no defence. The clinical features, the epidemic nature and the irregular periodicity all bring it in line with well-known bacterial diseases. Recently I have described amyloid degeneration in four cases of long standing subacute rheumatism. These cases were very typical, pure cases of rheumatism and neither in the clinical history nor in the histological examination of the organs after death was there anything to suggest any of the ordinary causes of amyloid degeneration.

Though it is true that the causes of amyloid degeneration are not clearly understood, yet it is generally recognized that the condition is secondary to some infective agent, and that, most generally, bacterial. All the experimental evidence of Krakow, Maximow, Davidsohn and others goes to support the bacterial cause.

When, however, we come to deal with the specific bacterium of rheumatism we enter much more controversial fields. It seems to me that we can dismiss from our consideration all the organisms which have been described at various times as causal except the diplococcus of Wasserman, and that which has been isolated and described in England by Poynton and Paine,[1, 2, 3] Walker,[4] Saw,[5] and myself;[6] this bacterium is probably the same as that

described by Wasserman. By these observers this organism has been isolated from definite cases of acute rheumatism, has been cultivated outside the body, has produced in rabbits and monkeys polyarthritis, endocarditis and other manifestations of rheumatic fever; and has again been isolated from the lesions in these organs. The case at first sight seems very strong, but various objections have been lodged, and it is with these that I propose dealing here.

The organism isolated, it has been thought, is not causal, the infection being simply a terminal one. Had this objection not been raised in authoritative quarters it would have been hardly worth dealing with. Terminal bacterial infections are not by any means common; besides the organism has been isolated from patients during life and when they have not been specially ill. Again, if we get a terminal bacterial infection, we expect it to be general and would expect to find the causal organism in the blood. In the three cases which I have examined after death (two of them only a few hours after), culture tubes inoculated from the blood remained sterile, and cultures of the organism were got from isolated areas only in the synovial membranes.

Acute rheumatism, it is claimed, is simply an attenuated pyæmia, and the organism isolated is an ordinary Streptococcus pyogenes. This objection has been supported by many eminent bacteriologists and must therefore be examined in detail. Rheumatism in many respects does resemble pyæmia. Both exhibit themselves in arthritis, endocarditis, etc., but there are definite distinguishing features, and save perhaps in the early stages clinicians do not mistake the one condition for the other.

Considering the very severe symptoms, and often the rapidly fatal issue in cases of acute rheumatism it seems to me a travesty of terms to speak of it as an "attenuated pyæmia." Again in pyæmia, and especially where the organism is not very active, pus formation is the common result; with acute rheumatism pus formation is the exception. As will be seen from the subjoined experiments pus was quite common in arthritis following injections of various forms of streptococci, whereas in the arthritis following inoculation with *Micrococcus rheumaticus* pus was not got in a single case.

For purposes of comparison I have examined and carried out inoculation experiments with twelve strains of streptococci and three of the special rheumatic organism:

Streptococcus 1 was isolated from a case of cellulitis; *2*, from the pus in the knee joint in a case of pyæmia; *3*, from pus in the mastoid cells in a case of middle ear disease; *4*, from a similar case; *5*, from a pyæmic abscess in the region of the appendix following an operation on a septic knee joint; *6*, from the throat in a case of measles; *7*, from the throat of a patient with scarlet fever; *8* and *9*, from separate cases of diphtheria; *10*, from a case of measles; *11*, from an acute tonsillitis; and *12*, from a case of diphtheria.

Micrococcus rheumaticus was isolated from three cases of definite acute rheumatism. These organisms were all identical in their cultural characters, but as the majority of the inoculations were carried out with one strain only, it is taken as the standard throughout the paper.

MICROCOCCUS RHEUMATICUS.

Micrococcus rheumaticus was isolated from a case of acute rheumatism on December 9, 1904, and since that time it had been subcultured frequently, often, however, intervals of two months elapsing between the times of subcultivation. The history of the case and the results of the inoculation experiments have already been published[7] and need not be further referred to here.

Morphological Characters and Staining Reaction.—It may be stated as a general rule that the coccus we have isolated, and which for convenience we call *Micrococcus rheumaticus* or *Streptococcus rheumaticus* is in its morphological characters and staining reactions indistinguishable from strains of *Streptococcus pyogenes.*

Cultural Characters.—Micrococcus rheumaticus grows quite readily at the room temperature. The growth on gelatine at the room temperature is very definite in twenty-four hours, and much more copious than the growth of any of the varieties of streptococci which were used. The acid production is extremely marked in any of the ordinary media, but some of the strains of streptococci used gave quite as marked an acid reaction, and as will be seen in the tables below the acid production and the reactions in the various sugars gave no help in distinguishing the one class of organism from the other.

The only definite and very distinctive reaction was the production of acid and precipitation of the bile salts by *Micrococcus*

rheumaticus in McConkey's bile salt lactose broth. No reaction was got in this medium with any of the strains of streptococci used. This difference was so marked that the observations were repeated with exactly similar results. The vitality of the organism outside the body I have referred to in a previous publication, but this was most strikingly illustrated during these investigations. Constant subculture was necessary to keep the streptococci alive, and three of the strains were lost during the course of the investigations. With *Micrococcus rheumaticus* there was not the slightest difficulty; several months could elapse and quite active subcultures could be obtained both in blood agar and in ordinary agar.

	Raffinose.			Inulin.			Saccharose.			Salacin.			Mannite.		
	Hrs. 24	Dys. 2	Dys. 3	Hrs. 24	Dys. 2	Dys. 3	Hrs. 24	Dys. 2	Dys. 3	Hrs. 24	Dys. 2	Dys. 3	Hrs 24	Dys. 2	Dys. 3
Mic. rh.	o	x	x	o	o	o	o	(x)	x	x	x	x	o	o	o
Strepto. I.	o	o	o	o	o	o	o	o	o	o	o	(x)	o	o	o
" II.	o	o	o	o	o	o	o	x	x	o	o	o	o	o	o
" III.	o	o	o	o	o	o	o	o	o	o	o	x	o	o	o
" IV.	o	o	o	o	o	o	o	x	x	o	o	o	o	o	o
" V.	o	o	o	o	o	o	o	x	x	o	o	o	o	o	(x)
" VI.	o	o	o	o	o	x	o	o	o	o	o	o	o	o	o
" VII.	o	x	x	o	o	x	o	(x)	x	o	o	o	o	o	o
" VIII.	o	x	x	o	o	o	o	o	x	o	x	x	o	o	o
" IX.	o	x	x	o	o	(x)	o	x	x	o	x	(x)	o	o	o
" X.	x	x	x	o	o	(x)	o	x	x	o	o	x	o	o	o
" XI.	o	o	o	o	o	o	o	o	(x)	o	o	o	o	o	o
" XII.	o	x	x	o	o	o	o	x	x	o	x	x	o	o	o

x indicates acid change; o indicates a negative result. Brackets ( ) indicate feeble development of the change.

	Milk.			Litmus Milk.			Taurocholate Broth.			Neutral Red.		
	Hrs. 24	Dys. 2	Dys. 3	Hrs. 24	Dys. 2	Dys. 3	Hrs. 24	Dys. 2	Dys. 3	Hrs. 24	Dys. 2	Dys. 3
Mic. rh.	o	xc	xc	x	xc	xc	x	x	x	o	o	x
Strepto. I.	o	o	o	o	o	(x)	o	o	o	x	x	x
" II.	o	o	o	o	(x)	(x)	o	o	o	o	o	o
" III.	o	o	o	o	x	o	o	o	o	o	o	o
" IV.	o	o	o	o	o	o	o	o	o	o	o	o
" V.	o	o	o.	(x)	(x)	(x)	o	o	o	(x)	(x)	(x)
" VI.	o	(xc)	(xc)	x	x	x	o	o	o	o	o	o
" VII.	o	xc	xc	x	xc	xc	o	o	o	o	o	x
" VIII.	o	o	o	x	x	x	o	o	o	o	x	x
" IX.	o	xc	xc	x	xc	xc	o	o	o	o	o	x
" X.	o	xc	xc	xc	xc	xc	o	o	o	o	o	x
" XI.	o	o	o	x	x	x	o	o	o	o	o	x
" XII.	o	o	o	x	x	xc	o	o	o	o	o	o

Brackets ( ) indicate feeble development of the respective change. c indicates coagulation.

The following tables show the cultural reactions in sugars, etc., of the strains of streptococci examined and of *Micrococcus rheumaticus.*

	Glucose Broth.			Lactose Broth.			Maltose Broth.		
	Hrs. 24	Dys. 2	Dys. 3	Hrs. 24	Dys 2	Dys. 3	Hrs 24	Dys. 2	Dys. 3
Mic. rh.	x	x	x	x	x	x	x	x	x
Strepto. I.	x	x	x	(x)	x	x	x	x	x
" II.	x	x	x	x	x	x	x	x	x
" III.	No growth.			No growth.			No growth.		
" IV.	x	x	x	(x)	x	x	x	x	x
" V.	x	x	x	x	x	x	x	x	x
" VI.	x	x	x	x	x	x	x	x	x
" VII.	x	x	x	x	x	x	x	x	x
" VIII.	x	x	x	x	x	x	x	x	x
" IX.	x	x	x	(x)	x	x	o	x	x
" X.	x	x	x	x	x	x	x	x	x
" XI.	x	x	x	x	x	x	(x)	x	x
" XII.	x	x	x	x	x	x	x	x	x

INOCULATION WITH STREPTOCOCCUS.

Cultures for these experiments were made on ordinary sloped agar tubes and an emulsion was made in 0.85 per cent. saline solution immediately before inoculation. It has not been deemed necessary to deal in detail with the microscopic examination of the various organs, though this has been carried out in a considerable number of cases.

The tables appended give the main results necessary for comparative purposes.

In this table lesions such as are common to any septic infection, *e. g.*, cloudy swelling, etc., will not be referred to.

Organism.	Rabbit.	Date and Site of Injection	Quantity of Injection.	Date of Death.	Symptoms Noted During Life.	Post Mortem. Arthritis.	Post Mortem. Endocarditis.	Post Mortem. Other Lesions.
Strepto-coccus 1.	1	17 Jan., 1906, intravenous.	Half agar tube.	Killed, 5 Feb.	Swelling left wrist, 22 Jan.; fluctuation in left and right wrists with redness of skin, 30 Jan.	Purulent; in right and left wrists.	Nil.	Nil.
	2	17 Jan., 1906, right knee joint.	Half agar tube.	Killed, 5 Feb.	Tenderness, swelling, fluctuation in right knee.	Purulent; in right knee with infiltration with pus of tissues round.	Nil.	Nil.
	3	26 Feb. to 2 Apr., intra-peritoneally.	Two loops to one agar tube.	Killed, 2 Apr.	Nil.	Nil.	Nil.	Nil.
	4	4 June, intravenous.	One agar tube.	Killed, 11 July.	Ill for two days.	Nil.	Nil.	Nil.
	5	4 June, intravenous.	Two agar tubes.	Killed, 11 July.	"	Nil.	Nil.	Nil.
Strepto-coccus 2.	1	24 Jan., intra-venous.	Half tube.	Died, 4 Feb.	31 Jan., very ill, no evidence of joint affection.	Nil.	Nil.	Septicaemia; cultures from heart blood.
	2	24 Jan., intra-venous.	Half tube.	Died, 16 Feb.		Nil.	Nil.	Heart very much dilated; septicaemia.
	3	26 Feb. to 2 Apr. 7 injections intra-peritoneally.	Two loops to one tube.	Killed, 2 Apr.	Nil.	Nil.	Nil.	Nil.
	4	5 June, intra-venous.	Half tube.	Died, 10 June.	—	—	—	Septicaemia; (organisms in blood).

Organism.	Rabbit	Date and Site of Injection.	Quantity of Injection.	Date of Death.	Symptoms Noted During Life.	Post Mortem.		
						Arthritis.	Endocarditis.	Other Lesions.
Strepto-coccus 3.	1	17 Jan., subcutaneous.	One tube.	Killed, 2 Apr.	Pus formed at site of injection.	Nil.	Nil.	Nil.
	2	17 Jan., knee joint.	Half agar tube.	Killed, 2 Apr.	Swelling, tenderness and fluctuations in injected joint.	At death joint dislocated and fixed.	Nil.	Nil.
	Same rabbit (2)	3 Mar., intraperitoneally.	One agar tube.	—	Joint not altered by this injection.	—	—	—
	3	24 Jan., intravenously.	Half agar tube.	Killed, 2 Feb.	Lameness, swelling in right wrist, 30 Jan.; 31 Jan., fluctuation.	Purulent; right wrist.	Nil.	Nil.
	4	7 injections, 26 Feb. to 22 Mar. intraperitoneally.	Two loops to one tube.	Died, 23 Mar.	—	—	—	Septicaemia; streptococci in general circulation.
	5	4 June, intravenously.	One tube.	Killed, 27 June.	Nil.	Nil.	Nil.	Nil.
	6	4 June, intravenously.	One tube.	Killed, 27 June.	Nil.	Nil.	Nil.	Nil.
Strepto-coccus 4.	1	17 Jan., subcutaneous.	One tube.	Killed, 2 Apr.	Nil.	Nil.	Nil.	Nil.
	2	17 Jan., knee joint.	Half tube.	Killed, 15 Feb.	Swelling and fluctuation in injected knee.	Purulent; knee; erosion of bone; infiltration of tissues round knee.	Nil.	Nil.
	3	24 Jan., intravenously.	One tube.	Killed, 2 Apr.	Nil.	Nil.	Nil.	Nil.

Organism.	Rabbit.	Date and Site of Injection.	Quantity of Injection.	Date of Death.	Symptoms Noted During Life.	Post Mortem.		
						Arthritis.	Endocarditis.	Other Lesions.
Strepto-coccus 4.	4	26 Feb. to 2 Apr., 7 injections intra-peritoneally.	Two loops to one tube.	Killed, 2 Apr.	Nil.	Nil.	Nil.	Nil.
	5	30 May, intra-venously.	One tube.	Killed, 16 June.	6 June, lameness left fore limb.	Purulent; left wrist.	Nil.	Nil.
	6	30 May, intra-venously.	Two tubes.	Killed, 16 June.	6 June, tenderness and swelling left wrist; 12 June, fluctuation.	Purulent; left wrist.	Nil.	Nil.
Strepto-coccus 5.	1	16 Feb., intra-venously.	Fourth tube.	Killed, 22 Feb.	17 Feb., swelling and pain in right wrist; 22 Feb., fluctuation, pain and swelling in left wrist.	Purulent; right wrist; synovial membranes in left wrist congested; no pus.	Nil.	Nil.
	2	26 Feb. to 2 Apr., 5 injections intra-peritoneally.	Two loops to one tube.	Killed, 5 Apr.	Nil.	Nil.	Nil.	Nil.
	3	5 June, intra-venous.	One and one-half tubes.	Died, 7 June.	Nil.	Nil.	Nil.	Septicaemia; organisms in blood.
	4	5 June, intra-venous.	One and one-half tubes.	Killed, 16 June.	10 June, lame left fore limb; 12 June, fluctaution in left wrist.	Purulent; left wrist.	Nil.	Nil.
Strepto-coccus 6.	1	8 May, subcu-taneous.	One tube.	Killed, 3 July.	Nil.	Nil.	Nil.	Nil.

Organism	Rabbit.	Date and Site of Injection.	Quantity of Injection.		Symptoms Noted During Life.	Post Mortem.		
						Arthritis.	Endocarditis.	Other Lesions.
Strepto-coccus 6.	2	16 May intra-venous.	One tube.		Paralysis of both hind limbs, 22 May.	Nil.	Nil.	An abscess in lumbar region of the spinal canal pressing on the spinal cord.
This organism died before further experiments could be made with it.								
Strepto-coccus 7.	1	16 May, intra-venous.	One tube.	Killed, 3 July.	Nil.	Nil.	Nil.	Nil.
	2	27 June, intra-venous.	One tube.	Killed, 11 July.	Nil.	Nil.	Nil.	Nil.
	3	27 June, intra-venous.	Two tubes.	Killed, 11 July.	Nil.	Nil.	Nil.	Nil.
	4	15 June to 10 July, subcutaneous and intraperitoneal; 5 injections.	Half to one tube.	Killed, 24 July.	Nil.	Nil.	Nil.	Pus at the site of subcutaneous injection.
Strepto-coccus 8.	1	8 May, intra-venous.	One tube.	Killed, 3 July.	Nil.	Nil.	Nil.	Nil.
	2	16 May, intra-venous.	One tube.	Killed, 3 July.	Nil.	Nil.	Nil.	Nil.
	3	29 May, intra-venous.	Two tubes.	Killed, 3 July.	Nil.	Nil.	Nil.	Nil.
The organism died before further experiments could be made.								
Strepto-coccus 9.	1	16 May, intra-venous.	One tube.	Killed, 3 July.	Nil.	Nil.	Nil.	Nil.

Organism.	Rabbit.	Date and Site of Injection.	Post Mortem. Arthritis.	Endocarditis.	Other Lesions.
Streptococcus 9.	2	12 July, intravenous.	Nil.	Nil.	Nil.
	3	15 June to 10 July, subcutaneous and intravenously 5 injections.	Nil.	Nil.	Pus at the site of subcutaneous inoculation.
Streptococcus 10.	1	16 May, intravenous.	—	—	—
	2	25 May, intravenous.	Nil.	Aortic endocarditis; vegetations large; ulceration of valves (ulcerative endocarditis).	Abscess in spinal column pressing on lower part of spinal cord; pyaemic abscesses in kidney.
	3	18 June, intravenous.	Nil.	Nil.	Nil.
	4	26 June, intravenous.	Nil.	Nil.	Nil.
	5	15 June to 10 July, subcutaneous and intraperitoneally; 5 injections.	Nil.	Nil.	Pus at the site of subcutaneous inoculation.

Organism.	Rabbit	Date and Site of Injection.	Quantity of Injection.	Date of Death.	Symptoms Noted During Life.	Post Mortem.		
						Arthritis.	Endocarditis.	Other Lesions.
Strepto-coccus 11.	1	16 May, intra-venous; and 15 May to 10 July, subcutaneously and intraperitoneally.	One tube. Half to one tube.	Killed, 17 July.	15 Jun sis hind from com cove scess subc injec			Nil.
	2	25 May, intra-venous.	Two tubes.	Died, 1 June.	27 Ma ing dern wris			Nil.
	3	18 June, intra-venous.	One tube.	Killed, 25 June.	21 Ju ness limb ing wri cons oede			Nil.
Strepto-coccus 12.	1	16 May, intra-venous.	One tube.	Killed, 3 July.				Nil.
	2	20 May, intra-venous.	Two tubes.	Killed, 3 July.				Nil.
	3	28 June, intra-venous.	One tube.	Killed, 12 July.				Nil.
	4	15 June to 10 July, subcutaneous and intraperitoneal, 5 injections.	Half to one tube.	Killed, 24 July.				Abscess at site of subcutaneous inoculation.

Summary of Inoculations with Streptococcus.

Intravenous	34
Intraperitoneal	7
Subcutaneous	3
Both intraperitoneal and subcutaneous	4
Total	48
Number of deaths	7 or 14.5%
Number of animals with arthritis	9 or 18.7%
Number of animals with endocarditis	1 or 2.0%

In all cases the arthritis was definitely purulent. In animals injected subcutaneously pus formed at the site of injection.

In the single experiment with endocarditis, there was distinct ulceration of the valves and the organisms were invading the adjacent muscle of the heart. There were pyæmic abscesses in the kidney, and an abscess in the lower dorsal vertebra. This lesion was evidently pyæmic endocarditis, a condition which everyone admits may occur in the course of a septic infection whatever be the organism present.

INOCULATION WITH MICROCOCCUS RHEUMATICUS.

Rabbit 1.—Inoculated in the right knee on November 8, 1905; was killed on March 26, 1906. There was stiffness about the right knee, but otherwise the animal appeared to be in perfect health. On March 8 it received an intraperitoneal injection of one half tube of culture. The following day the knee was swollen and painful, but in four days the acute symptoms had passed off. It was reinoculated in the peritoneal cavity on March 15 and 22, and on each occasion the acute symptoms reappeared and subsided again. At the post-mortem examination the joint contained a very small quantity of viscid exudation, which on examination was mainly composed of large mononucleated cells. Polymorphonuclear leucocytes were present but were very few in numbers. There was considerable destruction of cartilage with numerous small superficial erosions of the bone. No organisms of any kind were found.

There was a small recent vegetation on one of the aortic cusps. No cultivations were obtained from it. The other organs showed nothing apart from the usual toxic changes.

Rabbit 2.—Inoculated on March 29 with two tubes intravenously. April 2 the animal was lame in the right fore limb. No swelling could be made out. The lameness became less, but the animal was killed on April 6. There was well-marked aortic endocarditis, the vegetations being about a sixteenth of an inch in diameter, and pure cultures of the organism were obtained from these. With the exception of very slight injection in the synovial membranes of the left elbow nothing abnormal could be made out in any of the joints.

Rabbit 3.—Inoculated intravenously with one tube of culture on March 29,

1906; showed lameness in the left fore limb on April 5, and was killed the following day. At the post-mortem examination there was very evident congestion in the synovial membranes in the left elbow. There was also a slight amount of congestion in the left hip joint. Tubes were inoculated from portions of the synovial membranes in the congested joints but no growth was obtained. There was no endocarditis.

Rabbit 4.—Inoculated intravenously with one tube of culture on May 24, 1906; showed no symptoms of any kind during life. It was killed on June 12, but nothing pathological was made out. There was no arthritis and no endocarditis.

Rabbit 5.—Inoculated intravenously with two tubes of cultures on May 24; showed no symptoms during life. It was killed on June 12. The mitral and tricuspid segments were distinctly thickened and though no distinct vegetations could be made out there was slight nodular thickening along the free edge of the tricuspid segments. There was a firm fibrous nodule about an eighth of an inch in diameter in the septum between the ventricles. On microscopic examination this showed well-marked interstitial myocarditis. In the kidneys were some small pin-point whitish areas seen under the capsule. On section these areas extended in a wedge into the kidney substance, some of them passing almost to the hilus. On microscopic examination they were seen to be composed of masses of small lymphoid cells, with very slight development of fibrous tissue.

These interstitial charges in the heart and kidney may have been accidental and independent of the injection, but in none of the other animals examined during this investigation was the same heart condition seen. The kidney lesion was observed in three animals and these had been inoculated with Micrococcus rheumaticus. In some recent experiments by Dr. Henry Wade of Edinburgh a number of dogs were inoculated with the well-known infective granuloma of the dog. In the great majority of these experiments the kidneys showed interstitial nephritis, and in the early stages the appearance was identical with that seen in these rabbits. The interstitial changes in the kidney of the dog are undoubtedly toxic in origin, and I am inclined to attribute the changes I have described also to toxines.

Rabbit 6.—Inoculated intravenously on May 30 with one tube of culture. No symptoms developed. The rabbit was killed on June 22. There was no evidence of arthritis. On the tricuspid segments there were some very recent, fairly firm, but rather doubtful vegetations. No organisms were found. There was marked œdema of the aortic segments, but no trace of endocarditis. On microscopic examination it was difficult to determine whether the deposit on the tricuspid segments was not merely blood clot.

Rabbit 7.—Inoculated intravenously on May 30 with two tubes of culture. No symptoms developed. The rabbit was killed on June 22. There was no evidence of arthritis or of endocarditis. In both kidneys there were numerous small dark wedge shaped areas depressed slightly below the surface. The naked eye appearance suggested infarctions. On microscopic examination the area examined was found to be composed of masses of small lymphoid-like cells, and was identical with the areas seen in Rabbit 5. No bacteria could be found in these areas.

Rabbit 8.—Inoculated intravenously on June 5 with three agar tubes of the culture. No symptoms developed, and the rabbit was killed on June 22. There was no arthritis, but on the anterior mitral valve segment there was a small recent vegetation. No culture tubes were inoculated from this vegetation, but on microscopic examination a few organisms, practically unaltered in appearance, were found just at the junction of the vegetation with the tissue of the valve.

Rabbit 9.—A young rabbit of 600 grams weight was inoculated intravenously on June 13 with the culture from four agar tubes. On June 17 the rabbit looked very ill, and could not be got to move about. It was impossible to decide as to the presence or absence of arthritis. The animal died on June 18 and at the post mortem examination a slight amount of serous fluid was found in both elbows, but there was no congestion of the synovial membranes. On microscopic examination the fluid contained some mononucleated cells, but hardly any polymorphonuclear leucocytes. No bacteria were detected. Cultures were negative. The synovial membranes of both knees were very markedly congested, and there was very evident dilatation of the vessels along the inner side of the condyles of both femora. There was a slight amount of clear fluid in the joint cavities. On microscopic examination there were a fair number of polymorphonuclear cells, but the large proportion of the cells were mononucleated. These were markedly vacuolated and showed abundant phagocytosis of polymorphonuclear cells.

Cultures in milk broth, made by inoculation with small pieces of the membranes, gave pure cultures of the organism, whereas those inoculated with the fluid in the joint remained sterile.

No vegetations were made out. Microscopic examinations of the heart blood showed no organisms, and cultures from it remained sterile. Microscopic examination of the synovial membranes showed irregular scattered areas of polymorphonuclear leucocytes and in these areas only were the organisms found (Fig. 1). This experiment is of extreme interest, for it seems to me to be in line with many cases of rheumatism. The organism is localized and produces its main effect by a toxin; it also proves that failure to obtain the organism from the blood or even from the joint exudation is no proof that the organism is not present.

Rabbit 10.—Inoculated intravenously with four tubes of culture on June 25; showed very definite lameness in the right fore limb on June 28, and on June 29 in the right hind limb. The rabbit recovered completely and was killed on July 18. Nothing abnormal could be made out on naked eye examination. Microscopic examination is not yet completed.

Rabbit 11.—Inoculated intravenously with four tubes of culture on June 25; showed very slight lameness in the right fore limb on June 28. No tenderness or swelling could be made out. The animal was quite well the following day. It was killed on July 18. Microscopic examination is not yet complete. No organisms were obtained in culture from the joints.

Rabbit 12.—Inoculated intravenously on June 26 with the growth on five culture tubes. On June 29 there was very definite lameness in the right hind limb. On July 1 there was very distinct improvement in the condition. On July 2 the animal was dragging the left hind limb also, and on July 18 when

Organism.	Rabbit.	Date and Site of Injection.	Quantity of Injection.	Date of Death.	Symptoms Noted During Life.	Post Mortem.		
						Arthritis.	Endocarditis	Other Lesions.
Micrococcus rheumaticus.	1	8 Nov., 1905 right knee joint.	A few loopsful.	26 Mar., 1906, killed.	Swelling and tenderness later, stiffness in right knee.	Non purulent with some destructive changes.	Very recent aortic.	
	2	29 Mar., intravenously.	Two tubes.	Killed, 8 Apr.	Lameness right fore limb.	Slight injection in synovial membrane of right elbow.	Well marked aortic.	
	3	29 Mar., intravenously.	One tube.	Killed, 6 Apr.	Lameness left fore limb.	Congestion of synovial membrane in left elbow.	Nil.	
	4	24 May, intravenously.	One tube.	Killed, 12 June.	Nil.	Nil.	Nil.	Nil.
	5	24 May, intravenously.	Two tubes.	Killed, 12 June.	Nil.	Nil.	Thickening of mitral and tricuspid segments.	Interstitial myocarditis; acute interstitial nephritis.
	6	30 May, intravenously.	One tube.	Killed, 22 June.	Nil.	Nil.	Doubtful vegetations on tricuspid and aortic segments.	Nil.
	7	30 May, intravenously.	Two tubes.	Killed, 22 June.	Nil.	Nil.	Nil.	Acute interstitial nephritis.
	8	5 June, intravenously.	Three tubes.	Killed, 22 June.	Nil	Nil.	Recent vegetation on mitral segment.	Nil.
	9	13 June, intravenously.	Four tubes.	Died, 18 June.	Indefinite, but animal acutely ill.	Acute in both knees.	Nil.	Nil.

Organism.	Rabbit.	Date and Site of Injection.	Quantity of Injection.	Date of Death.	Symptoms Noted During Life.	Post Mortem.		
						Arthritis.	Endocarditis.	Other Lesions.
Micrococcus rheumaticus.	10	25 June, intravenously.	Four tubes.	Killed, 18 July.	Lameness in right fore and right hind limb (recovered).	Nil.	Nil.	Examination not completed.
	11	25 June, intravenously.	Four tubes.	Killed, 18 July.	Lameness in right fore limb (recovered).	Nil.	Nil.	Examination not completed.
	12	26 June, intravenously.	Five tubes.	Killed, 18 July.	Lameness right hind limb; partial recovery.	Congestion of synovial membranes on both hips.	Nil.	Acute interstitial nephritis.
	13	10 July, intravenously.	Four tubes.	Killed, 13 July.	Indefinite; animal acutely ill.	Right knee.	Nil.	Septicaemia.
	14	10 July, intravenously.	Two tubes.	Died, 13 July.	Indefinite, but acutely ill.	Both knee joints.	Minute vegetations in mitral segments.	Septicaemia.
	15	10 July, subcutaneously.	Five c.c. broth culture.	Killed, 24 July.	Nil.	Nil.	Nil.	Nil.

it was killed, both hind limbs were weak and they appeared to be paralyzed. When the animal was allowed to run about it could move its limbs fairly well.

At the post mortem examination there was definite congestion of the ligamentum teres and synovial membranes generally in both hips. Nothing abnormal was made out in any of the other joints. There was no endocarditis. In the kidney there were several small wedge-shaped areas resembling infarctions. These were dark red and were depressed slightly below the surface of the organ. Microscopically these areas corresponded with those described in Rabbits 5 and 7.

Rabbit 13.—Inoculated intravenously on July 10 with the growth from four culture tubes. The following day the animal was evidently ill, but no arthritis could be made out.

On July 13 it was killed. There was slight injection of the synovial membrane in the right knee, and microscopic examination showed the presence of polymorphonuclear and mononucleated cells and also bacteria similar to Micrococcus rheumaticus.

Cultures from the heart blood gave a pure growth of the micrococcus. There were no vegetations.

Rabbit 14.—Inoculated intravenously on July 10 with the growth from two agar tubes. On July 11 it was very ill, and was found dead in its cage on July 13. There was congestion in the synovial membranes of both knee joints, and from the right pure cultures of the micrococcus were obtained. A pure culture was also obtained from the blood in the heart. There were numerous minute vegetations along the free margin of the mitral valve segments. On microscopic examination the vegetations were very definite and there was infiltration of the tissue of the valve with polymorphonucleated and mononucleated cells.

Rabbit 15.—Inoculated subcutaneously with 5 c.c. of a broth culture of Micrococcus rheumaticus on July 10, 1906. No pus developed at the site of inoculation. The rabbit was killed on July 24. No pathological lesions were detected.

Summary of Inoculations with Micrococcus Rheumaticus:

Intravenous	13
Into knee joint	1
Subcutaneous	1
Total	15
Number of deaths	2 or 13.3%
Number of animals with arthritis	9 or 60.0%
Number of animals with endocarditis (including one doubtful case)	5 or 33.3%

In all the experiments the arthritis was non-purulent, and the knee joints were frequently affected. Recovery or improvement took place if the animal was allowed to live. I would also call special attention to the presence of acute interstitial nephritis and myocarditis in the rheumatic cases. These were not seen in any

of the streptococcal cases. Their significance I am not yet in a position to state.

AGGLUTINATION.

By this means also differentiation is made out between the various forms of streptococci and *Micrococcus rheumaticus*. These reactions were all done the same day with similar solutions, and the results were checked by the independent observations of Dr. J. W. Dawson, to whom I am indebted for very much help during the course of these investigations.

The Agglutination was tested in sedimentation tubes. Dilution was 1 in 30.
Reaction xx = Definite Clumping.

I. (*Control*) *Emulsions alone:* In peptone broth of the separate organisms gave the following reactions:

Micrococcus rheumaticus	A few clumps.
Streptococcus I	No clumps or very few.
Streptococcus II	No clumps or very few.
Streptococcus III	No clumps or very few.
Streptococcus IV	No clumps or very few.
Streptococcus V	A few clumps.

II. (*Control*) *Normal Rabbit Serum:* Plus emulsion in peptone broth of the separate organisms gave the following reactions:

Micrococcus rheumaticus	Hardly any change.
Streptococcus I	Nil.
Streptococcus II	Nil.
Streptococcus III	Nil.
Streptococcus IV	Nil.
Streptococcus V	A few clumps but not nearly so marked as in immune serum.

III. Serum of Rabbit Immunized with Streptococcus I.

Plus emulsion in peptone broth of M.rh.	x ?	Very few clumps
Plus emulsion in peptone broth of Strepto. I	xxx	
Plus emulsion in peptone broth of Strepto. II	x	
Plus emulsion in peptone broth of Strepto. III	xxx	
Plus emulsion in peptone broth of Strepto. IV	xx	
Plus emulsion in peptone broth of Strepto. V	xxx	

IV. Serum of Rabbit Immunized with Streptococcus II.

Plus emulsion in peptone broth of M.rh.	x ?
Plus emulsion in peptone broth of Strepto. I	xxx
Plus emulsion in peptone broth of Strepto. II	xx
Plus emulsion in peptone broth of Strepto. III	xx
Plus emulsion in peptone broth of Strepto. IV	xx
Plus emulsion in peptone broth of Strepto. V	xxxx

V. Serum of Rabbit Immunized with Streptococcus III.

Plus emulsion in peptone broth of M.rh.	x	
Plus emulsion in peptone broth of Strepto. I	xxx	
Plus emulsion in peptone broth of Strepto. II	xx	
Plus emulsion in peptone broth of Strepto. III	xxxx	
Plus emulsion in peptone broth of Strepto. IV	xx	
Plus emulsion in peptone broth of Strepto. V	xxx	

VI. Serum of Rabbit Immunized with Streptococcus IV.

Plus emulsion in peptone broth of M.rh.	x	Very slight; one large clump at bottom of tube.
Plus emulsion in peptone broth of Strepto. I	xx	
Plus emulsion in peptone broth of Strepto. II	x	
Plus emulsion in peptone broth of Strepto. III	xx	
Plus emulsion in peptone broth of Strepto. IV	xx	But some very large clumps.
Plus emulsion in peptone broth of Strepto. V	xx	

VII. Serum of Rabbit Immunized with Streptococcus V.

Plus emulsion in peptone broth of M.rh.	Nil.	
Plus emulsion in peptone broth of Strepto. I	xx	
Plus emulsion in peptone broth of Strepto. II	x	
Plus emulsion in peptone broth of Strepto. III	xxx	
Plus emulsion in peptone broth of Strepto. IV	x	Very slight.
Plus emulsion in peptone broth of Strepto. V	x	

VIII. Serum of Rabbit Immunized with Micrococcus rheumaticus.

Plus emulsion in peptone broth of M.rh.	xx	Big clumps; bigger than with Strepto. I.
Plus emulsion in peptone broth of Strepto. I	xx	
Plus emulsion in peptone broth of Strepto. II	Nil.	
Plus emulsion in peptone broth of Strepto. III	x	
Plus emulsion in peptone broth of Strepto. IV	Nil.	
Plus emulsion in peptone broth of Strepto. V	x ?	

The absence of the organisms from the blood and joint exudations during life. Poynton and Paine and Walker and Beaton record quite frequently successful cultivations from the blood and the exudates in the joints during life. Philipp,[8] in twenty-four cases of acute articular rheumatism, attempted cultivations twenty-one times from the blood and six times from the joint exudate, and twice from the blood and twice from the joints in chronic rheumatism. No bacteria were cultivated, though various kinds of media were used. Cole[9] reports that for the last three years, in prac-

tically all cases of acute rheumatism treated in the Johns Hopkins Hospital, routine cultures have been made from the blood and from the joints whenever any effusion was present. All the cultures were negative.

My own experience in this connection has been very limited. I have examined three cases of acute rheumatism—and all of them post-mortem. In all of them tubes inoculated from the blood remained sterile. In only one case cultures were made from the exudate in the joint, and these also were negative. In the three cases, however, the organism was grown from pieces of the synovial membrane. Several of the tubes inoculated with pieces of synovial membrane also remained sterile.

The results of my culture experiments, and also of microscopic examination, indicate that the organism may be missed altogether in cases where the arthritis is the principal manifestation of the disease, unless several different areas of the synovial membranes are examined.

No doubt in some cases, and especially in severe attacks and those with vegetative endocarditis, the organisms may be found in the blood at certain stages of the disease, but in most of the ordinary cases the organisms appear to be localized and probably produce their results by a toxin. Unless these localized areas are examined there is no possibility of getting cultivations.

Rabbit 9 is of extreme interest in this connection as in it has been reproduced a condition which is the common one in acute rheumatism in the human subject, *i. e.*, a localized bacterial infection in the synovial membranes and secondary effects the result of a toxine. By definite experiment, I have thus shown a complete picture of a case of infection with *Micrococcus rheumaticus* where joint exudation and blood are sterile. Unfortunately, I have, through want of time, not yet been able to repeat this experiment.

Further, the "interstitial nephritis" seen in several of the rabbits, shows the secondary results of the toxine. This also came under my notice late in the research, and has opened up a field which will require careful investigation before any definite conclusion can be drawn from it.

CONCLUSION.

The conclusions I would draw from this work are merely those stated in a former paper.

1. The results obtained by injections of streptococci are different from those produced by *Micrococcus rheumaticus.*

2. *Micrococcus rheumaticus* cannot be regarded as an attenuated streptococcus, nor acute rheumatism as an attenuated streptococcal pyæmia.

3. In uncomplicated cases of acute rheumatism the organism may not be found in the blood or in the joint exudates.

EXPLANATION OF PLATE VI.

FIGURE 1. Synovial Membrane (Rabbit 9) showing localized inflammatory areas (*a*) in which the bacteria were found.

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Fig. 1.

LEUCOPROTEASE AND ANTI-LEUCOPROTEASE OF MAMMALS AND OF BIRDS.

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Studies of Fr. Müller[1] and subsequent observers have shown that the polynuclear leucocytes of man and other mammals contain an enzyme which digests proteid and is particularly active in the presence of a weak alkaline reaction. One of us[2] has shown that a suspension of cells from an inflammatory exudate causes proteolysis both in an alkaline and in an acid medium, though digestion is more active in the former. It has further been possible to obtain from such cells a dry powder[3] which digests only in the presence of an alkaline reaction; this enzyme has been designated leucoprotease.

Proteolysis caused by cells of an inflammatory exudate in the presence of acid is more active in proportion to the number of large mononuclear phagocytes or macrophages which are present, while an emulsion made from the lymphatic glands which are situated near the seat of inflammation and contain such cells in immense number causes proteolysis in an acid medium and fails to digest in a neutral or alkaline solution; for convenience, this enzyme has been designated lympho-protease. Hence, each of the two types of phagocytic cells which are capable of ingesting and dissolving within their substance micro-organisms and other proteid-containing bodies is characterized by an enzyme, namely, the polynuclear leucocyte contains leucoprotease, which digests in a neutral or alkaline medium while the large mononuclear phagocyte, which is most abundant in the inflammatory exudate during the later stages of inflammation and often attacks and digests the smaller polynuclear cell, contains lymphoprotease which digests only in the presence of acid.

[1] Kossel, *Zeit. f. klin. Med.*, 1888, xiii, 149.
[2] *Jour. of Exper. Med.*, 1905, vii, 316.
[3] *Ibid.*, 1906, viii, 410.

Leucoprotease, like trypsin, digests in the presence of an alkaline reaction, while lympho-protease, like pepsin, requires an acid medium. Lymphoprotease is active in the presence of very weak hydrochloric acid (1/100 N.) but fails to act in the presence of that concentration of hydrochloric acid which is most favorable to the action of pepsin.[4] Leucoprotease, moreover, has been found to be far less active than trypsin. Further knowledge concerning the relation of the enzymes of leucocytes to the enzymes of the digestive tract is wanting.

The studies of Salkowski[5] have shown that organs kept at body temperature under conditions which prevent bacterial growth undergo self-digestion. Bondi[6] found that self-digestion of liver tissue is more active in an acid than in an alkaline medium. Hedin and Rowland[7] have shown that juice expressed from the spleen of the beef, horse, pig and sheep undergo especially active autolysis in the presence of an acid reaction. The reaction of the expressed juice is acid, but the addition of 0.1 per cent. hydrochloric acid increases proteolysis. In the presence of an alkaline reaction produced by adding from 0.2 to 0.37 per cent. of sodium bicarbonate, the degree of autolysis is diminished, but is, nevertheless, considerable. Subsequent observations[8] have shown that lymphatic glands, the kidneys and the liver contain proteolytic enzymes which are more efficient in an acid than in an alkaline medium. The juice expressed from voluntary muscle undergoes only slight autolysis which is not increased either by addition of acid or of alkali. The heart muscle contains an enzyme which resembles that of other organs and is more active in the presence of acid. Levene and Stookey[9] found that autolysis of nerve tissue and of testis is increased by acid.

Hedin[10] succeeded in separating from the spleen two enzymes, one of which, designated by him lieno-α-protease, acted in alkaline

[4] *Ibid.*, 1906, viii, 418.
[5] *Zeit. f. klin. Med.*, 1890, Suppl. zum xvii, 77.
[6] *Virchow's Archiv.* 1896, cxliv, 373.
[7] *Zeit. f. physiol. Chem.*, 1901, xxxii, 341.
[8] *Ibid.*, 1901, xxxii, 531.
[9] *Jour. Med. Research*, 1903, x, 212.
[10] *Jour. of Physiol.*, 1904, xxx, 155.

medium, while a second enzyme, which he called lieno-β-protease, acted in the presence of acid. After spleen pulp had been digested in the presence of 0.1–0.2 per cent. acetic acid, that enzyme which digested in the presence of acid was found in solution, while from the residue, after extraction with three per cent. sodium chloride, precipitation with acetic acid and subsequent neutralization, an enzyme was obtained which acted rather strongly in an alkaline medium and much less in an acid. Hedin showed that the enzymes which he isolated not only caused autolysis, by digesting the substance of the cells which contained them, but were capable of breaking down other proteids, such as fibrin, casein and coagulated blood serum.

The cells of an inflammatory exudate obtained by injecting aleuronat into the pleural cavity of the dog, unlike all of the organs which have been mentioned cause more active proteolysis in an alkaline than in an acid medium. Another tissue has been found by one of us[11] to share this property, namely, the bone-marrow. A suspension of cells from the spleen, lymphatic glands, liver or kidneys caused much more active proteolysis of heated serum with acid than with alkali, while similarly prepared suspensions of cells from the bone-marrow were far more active in an alkaline medium. It is not improbable that the enzyme which is present in large amount in the cells of the exudate rich in polynuclear leucocytes and in the tissue from which the polynuclear lucocytes arise is identical with the similar enzyme which Hedin obtained from the spleen.

The Relation of Anti-leucoprotease to the Globulins and Albumin of the Blood Serum.—Hahn[12] first showed that normal blood serum has the power of inhibiting or wholly preventing the action of trypsin. According to Landsteiner[13] this anti-enzymotic action is not possessed by the serum globulin, precipitated by half saturation with ammonium sulphate, but is present in the albumin precipitated by complete saturation with ammonium sulphate after removal of the globulin. Glaessner[14] failed to confirm this observation, main-

[11] *Jour. of Exper. Med.*, 1905, vii, 759.
[12] *Berliner klin. Woch.*, 1897, xxxiv, 499.
[13] *Cent. f. Bakt.*, 1900, xxvii, Abt. i, 357.
[14] *Hofmeister's Beiträge*, 1904, iv, 79.

taining that the euglobulin fraction precipitated by one third saturation with ammonium sulphate inhibited the action of trypsin on coagulated proteid contained in Mett's tubes, while the pseudo-globulin subsequently precipitated by half saturation exhibited little, and the albumin fraction, no anti-enzymotic action. The results obtained by Landsteiner have been confirmed by Cathcart,[15] who found anti-tryptic action with the albumin, but not with the globulin fraction. This anti-enzymotic action of the serum is destroyed by a temperature of 70° C. but that of the isolated albumin fraction is destroyed by 55° C.

That enzyme of the spleen which acts in an alkaline medium and has been designated by Hedin lieno-α-protease is inhibited by the blood serum. Hedin found that the substance in the serum of the ox which checked this enzyme was contained in the albumin and pseudo-globulin fractions, whereas, in one experiment the euglobulin fraction slightly increased, in another slightly diminished, its activity. That part of the euglobulin fraction which was precipitated by dialysis of the serum was found to increase rather than diminish proteolysis caused by the splenic enzyme.

It has been shown by one of us[16] that proteolysis in an alkaline or approximately neutral medium caused by a suspension of cells from an inflammatory exudate is prevented by small quantities of the serum of the exudate or of the serum of the blood. This anti-enzymotic action is destroyed by a temperature of 75° C. but is unaffected by heating to 70° C. during an half hour. Baer and Loeb[17] observed that the serum of the blood exerted a similar action upon the autolytic enzyme contained in the liver, but since, as they believed, this property was little, if at all, altered by heat, even at the temperature of boiling, they did not think it attributable to a true anti-enzyme. The same inhibiting action was exerted by the albumin of the serum but was wholly lacking in the globulin.

The purpose of the following experiments has been to determine primarily if the power to resist the action of the leucoprotease of the polynuclear leucocytes is common to all proteids of the blood

[15] *Jour. of Physiol.*, 1904, xxxi, 497.
[16] *Jour. of Exper. Med.*, 1905, vii, 316.
[17] *Arch. f. exper. Path. u. Phar.*, 1905, liii, 1.

serum or if this anti-body is localized in a particular fraction of the serum. The somewhat discordant results obtained with anti-bodies for other proteolytic enzymes have been cited for comparison.

To separate the globulins and albumin of the blood, the method of fractioning the serum employed by Freund and Joachim[18] has been used. The blood serum after centrifugalization was diluted with three times its volume of distilled water. A small amount of precipitate, euglobulin, was obtained by adding a saturated solution of ammonium sulphate in quantity to cause one third saturation. The precipitate was washed, dissolved in distilled water, and dialyzed; it was again precipitated by one third saturation with ammonium sulphate and again dialyzed. To the filtrate obtained after one third saturation was added a saturated solution of ammonium sulphate in quantity sufficient to cause one half saturation. The bulky precipitate thus obtained was dissolved in water, dialyzed and reprecipitated. The filtrate was completely saturated with ammonium sulphate by the addition of dry salt. A bulky precipitate, albumin, was obtained and further purified as before. The fractions of serum obtained by this method were dissolved in a volume of water approximately equal to or in some instances double that of the serum employed.

Dried and powdered leucocytes prepared by the method previously described (leucoprotease), in weighed quantities, were allowed to act at 37° C. during five days on a measured quantity of coagulated proteid (five cubic centimeters of heated serum) in the presence of the various fractions which had been isolated. Digestion took place in closely stoppered flasks; the volume of each mixture was brought to twenty-five cubic centimeters by addition of 0.85 per cent. salt solution, and one cubic centimeter of toluol was added. The amount of nitrogen in substances incoagulable by heat has been measured by the Kjeldahl method, and for the sake of comparison is given in terms of cubic centimeters of 1/10 N. sulphuric acid. Since previous experiments had shown that the anti-enzymotic activity of the serum was slightly greater when the alkalinity of the medium was increased, to each mixture in the following experiment was added 0.2 per cent. of sodium carbonate;

[18] *Zeit. f. physiol. Chem.*, 1903, xxxvi, 407.

in subsequent experiments this addition was not made. Former experiments having shown that the anti-enzymotic action of the serum is destroyed by heat, the effect of the unheated fraction was compared with that of the same fraction previously heated to 75° C. during one half hour.

20 mgr. leucoprotease+coagulated proteid+10 c.c. euglobulin	20.5 c.c.
20 mgr. leucoprotease+coagulated proteid+10 c.c. euglobulin heated	16.1 c.c.
20 mgr. leucoprotease+coagulated proteid+10 c.c. pseudo-globulin	27.2 c.c.
20 mgr. leucoprotease+coagulated proteid+10 c.c. pseudo-globulin heated	21.05 c.c.
20 mgr. leucoprotease+coagulated proteid+10 c.c. albumin	13.8 c.c.
20 mgr. leucoprotease+coagulated proteid+10 c.c. albumin heated	29.7 c.c.

In order to test the action of the various fractions in the absence of leucoprotease, quantities of pseudo-globulin and of albumin equal to those previously employed were allowed to act on heated serum under conditions similar to those just described, addition of alkali being omitted. The control represents the amount of nitrogen in uncoagulable form contained in a given mixture before digestion.

10 c.c. pseudo-globulin	+ coagulated proteid	21.1 c.c.
Control		6.95 c.c.
10 c.c. albumin	+ coagulated proteid	10.8 c.c.
Control		9.6 c.c.

The experiment demonstrates that digestion is increased when the euglobulin and pseudo-globulin fractions are added to a mixture of leucoprotease and heated serum. Digestion is inhibited on the contrary by the albumin fraction. The second half of the experiment shows that the pseudo-globulin fraction contains an active proteolytic enzyme, while the albumin fraction fails to cause noteworthy digestion.

The following experiments confirm the results of that just described. The conditions of the experiments are the same, save that euglobulin and pseudo-globulin were not separated but were precipitated together by half saturation with ammonium sulphate. The globulin fraction was dissolved in a volume of water approximately equal to that of the serum from which it was obtained. The albumin fraction was dissolved in twice its volume of water.

20 mgr. leucoprotease + coagulated proteid + 15 c.c. globulin	33.45 c.c.
20 mgr. leucoprotease + coagulated proteid + 15 c.c. globulin heated	24.2 c.c.
20 mgr. leucoprotease + coagulated proteid + 15 c.c. albumin	7.5 c.c.
20 mgr. leucoprotease + coagulated proteid + 15 c.c. albumin heated	18.1 c.c.

The action of the two fractions on coagulated proteid in the absence of leucoprotease was further tested.

Coagulated proteid + 15 c.c. globulin	28.1 c.c.
Control	10.3 c.c.
Coagulated proteid + 15 c.c. albumin	7.05 c.c.
Control	6.55 c.c.

The globulin and albumin fractions used in the next experiment were dissolved in volumes of water approximately equal to that of the serum from which they were obtained.

20 mgr. leucoprotease + coagulated proteid + 5 c.c. globulin	25.2 c.c.
20 mgr. leucoprotease + coagulated proteid + 5 c.c. globulin heated	23.85 c.c.
20 mgr. leucoprotease + coagulated proteid + 5 c.c. albumin	13.1 c.c.
20 mgr. leucoprotease + coagulated proteid + 5 c.c. albumin heated	22.6 c.c.

The same quantities of the globulin and of the albumin were allowed to act upon coagulated proteid at 37° C. during five days in the presence of an approximately neutral, alkaline, and acid reaction:

Coagulated proteid + 5 c.c. globulin	16.3 c.c.
Coagulated proteid + 5 c.c. globulin + 0.2 per cent. sodium carbonate	6.5* c.c.
Coagulated proteid + 5 c.c. globulin + 0.2 per cent. acetic acid	3.7 c.c.
Control	2.3 c.c.
Coagulated proteid + 5 c.c. albumin	2.4 c.c.
Coagulated proteid + 5 c.c. albumin + 0.2 per cent. sodium carbonate	2.55 c.c.
Coagulated proteid + 5 c.c. albumin + 0.2 per cent. acetic acid	2.15 c.c.
Control	2.4 c.c.

* Since this figure differed markedly from that obtained when the reaction of the medium remained approximately neutral (16.3 c.c.) it was suspected that an error had occurred in making the Kjeldahl determination; repetition of the test gave the figure, 13.2 c.c.

These experiments which show that the anti-enzyme for leucoprotease is present in the albumin of the serum and absent in the globulins are analogous to those of Landsteiner and of Cathcart who found anti-trypsin only in the albumin fraction of the serum. Hedin, it has been mentioned, found an anti-body for his lieno-α-protease in the albuimn fraction, but present also, according to his observations, in the pseudo-globulin.

The globulin of the serum not only possesses no anti-leucoprotease but contains a proteolytic enzyme which is active under conditions similar to those which favor the action of leucoprotease. Delezenne and Pozerski[19] had observed that serum treated with chloroform

[19] *Compt. rend. Soc. de Biol.*, 1903, lv, 327, 690, 693.

digests proteid, and believed that the serum contained an anti-body which normally held this enzyme in check. Hedin[20] showed that a weak proteolytic enzyme which digests in an alkaline medium is present in the globulin of the serum and is inhibited by an anti-body which is mainly contained in the albumin. It is not improbable that this enzyme is identical with that which is present in the polynuclear leucocytes and in the bone marrow and with the similar enzyme of the spleen. In the blood serum this enzyme is held in check by the anti-body which is precipitated with the albumin; this anti-body which in given quantity doubtless holds in check only a limited quantity of enzyme,[21] is present in the serum in excess, so that the whole serum is capable of further anti-enzymotic action.

Action of Anti-leucoprotease of Different Mammalian Species upon Leucoprotease of the Same and of Different Species.—By study of the anti-enzymotic action of sera from animals of various species upon leucoprotease derived from different animals, it was hoped that evidence concerning the identity or multiplicity of such enzymes might be obtained; for if each of two enzymes bears a specific relationship to its own serum, it is improbable that the two enzymes are identical. Glaessner believed that the anti-tryptic action of the blood serum bears a specific relation to trypsin from the same species; he found that trypsin from the ox was more strongly inhibited by ox serum and trypsin from the pig by pig's serum than by other sera. The experiments of Cathcart[22] did not confirm this view, but were not decisive.

In order to test the inhibiting action of sera obtained from a variety of species upon the leucoprotease of the dog, a measured quantity (twenty milligrams) of the dry powder prepared from leucocytes, obtained either by injecting aleuronat into the pleural cavity or turpentine into the subcutaneous tissue, was allowed to act during five days at 37° C. upon a measured quantity of coagulated proteid in the presence of various sera. In the following experiment three cubic centimeters of the serum of dog, man, and ox were employed. To determine for comparison the proteolytic activity of

[20] *Jour. of Physiol.*, 1904, xxx, 195.
[21] *Jour. of Exper. Med.*, 1906, viii, 538.
[22] *Loc. cit.*

the enzyme, twenty milligrams were allowed to act upon coagulated proteid in the presence of three cubic centimeters of the various sera previously heated to 75° C. during one half hour, in order to destroy their anti-enzymotic action. The control represented the amount of nitrogen in uncoagulable substances present in the mixtures before digestion.

	Human serum.	Dog's serum.	Ox's serum.
Control	3.35 c.c.	4.65 c.c.	3 c.c.
With 3 c.c. heated serum	25.25 c.c.	26.5 c.c.	21.8 c.c.
With 3 c.c. unheated serum	7.4 c.c.	8.7 c.c.	7.6 c.c.

The sera of man and of ox act like that of the dog, and hinder digestion caused by the dog's enzyme. It is not improbable that a maximum degree of inhibition has been caused by these sera when three cubic centimeters of each were employed. In order to test more accurately the relative activity of the different sera, smaller quantities were employed; 0.5 and 0.25 cubic centimeters of the serum of dog, cat, goat, and pigeon were added to mixtures containing twenty milligrams of leucoprotease and a measured quantity of coagulated proteid.

	Dog's serum.	Cat's serum.	Goat's serum.	Pigeon's serum.
With 0.5 c.c. serum	9.9 c.c.	4.3 c.c.	4.75 c.c.	16.8 c.c.
With 0.25 c.c. serum	10.85 c.c.	4.25 c.c.	6.4 c.c.	17.6 c.c.

The proteolysis caused by twenty milligrams of the enzyme acting on the quantity of coagulated proteid used in the above mixtures is represented by 18.2 c.c., the control being 3.0 c.c.

The serum of the cat causes more complete inhibition of the dog's enzyme than the serum of the goat, but both sera are more actively anti-enzymotic than the dog's own serum. Digestion in the presence of equal quantities of pigeon's serum is little less than that caused by the unrestrained enzyme. In the following experiment the sera of dog and rabbit were compared, the effect of increasing quantities of these sera on twenty milligrams of dog's leucoprotease being tested. Since the coagulated proteid used for digestion (five cubic centimeters of heated dog's serum) was not the same in the two series, the results are not accurately comparable, but demonstrate that the inhibiting power of the rabbit's serum is considerably greater than that for the dog.

	Dog's serum.	Rabbit's serum.
With 0.25 c.c. serum	22.15 c.c.	7.35 c.c.
With 0.5 c.c. serum	18.8 c.c.	5.35 c.c.
With 1 c.c. serum	10.6 c.c.	4.3 c.c.
With 2.5 c.c. serum	7.55 c.c.	4.9* c.c.
Without the addition of serum	24.2 c.c.	21.45 c.c.
Control	2.85 c.c.	2.85 c.c.

* This figure is larger than that obtained when a smaller quantity of rabbit's serum was used because the mixture before digestion contained more nitrogen in uncoagulable form, 2.5 c.c. of dog's serum being represented by 1.45 c.c. and 2.5 c.c. rabbit's serum by 1.75 c.c.

In the second experiment the figures obtained are comparable, the same coagulated proteid being used for digestion in all of the tests.

	Dog's serum.	Rabbit's serum.
With 0.25 c.c. serum	12.5 c.c.	6.45 c.c.
With 1.0 c.c. serum	6.5 c.c.	5.15 c.c.

Digestion caused by the enzyme used in this experiment, in the absence of serum, is represented by 20.05 c.c., the control being 4.05 c.c.

Since the enzyme of the dog's leucocytes is more markedly inhibited by the serum of man, ox, cat, goat, and rabbit than by the dog's own serum, it is necessary to determine if the corresponding enzyme of other animals bears the same relation to foreign mammalian sera. For this purpose, the attempt was made to obtain polynuclear leucocytes of the rabbit in quantities sufficient for the tests required. By injection of aleuronat into the pleural cavity of the rabbit, only a small quantity of exudate was obtained, and this exudate was so poor in cells that their suspension in salt solution completely failed to digest coagulated proteid. Injections of turpentine into the subcutaneous tissue of the rabbit caused exudation of serum and accumulation of dry friable material of opaque white color forming a layer adherent to the necrotic tissues which had been in contact with the injected turpentine. This material consisted in great part of polynuclear leucocytes held together by fibrin; typical suppuration with softening and solution of tissue was entirely lacking. The exudate was scraped from the surface of the exposed tissues and dried by the method previously described. The dry powder thus obtained was found to have weak proteolytic action when allowed to act at body temperature on heated serum. The anti-

enzymotic action of rabbit's, dog's and hen's serum was tested with this enzyme.

20 mgr. leucoprotease + coagulated proteid + 0.5 c.c. rabbit's serum	3.1 c.c.
20 mgr. leucoprotease + coagulated proteid + 0.5 c.c. dog's serum	3.7 c.c.
20 mgr. leucoprotease + coagulated proteid	4.15 c.c.
Control	2.5 c.c.

Since the amount of digestion in the experiment, even in the absence of serum, was insignificant, a larger quantity of the ferment was employed.

50 mgr. leucoprotease + coagulated proteid + 0.5 c.c. rabbit's serum	3.45 c.c.
50 mgr. leucoprotease + coagulated proteid + 0.5 c.c. dog's serum	4.05 c.c.
50 mgr. leucoprotease + coagulated proteid	7.6 c.c.
Control	2.45 c.c.

These two experiments have shown that leucoprotease derived from the rabbit bears to the two sera with which it has been tested a relation which is identical with that of dog's leucoprotease to the same sera; the enzyme from both animals is inhibited in greater degree by the serum of the rabbit than by that of the dog. Since the anti-body of one species has no specific relation to the leucoprotease of the same species, the experiments tend to support the belief that the leucoprotease of different mammalian species is identical.

The enzyme from the rabbit is much weaker than the similarly prepared enzyme from the dog; the anti-enzyme of the rabbit's serum is, on the contrary, much more active. Since experiments previously described have shown that suppuration with solution of fibrin and of necrotic tissue in the dog is associated with loss of anti-enzyme in the purulent exudate, it is not improbable that the well-known absence of typical suppuration with liquefaction of tissue in the rabbit is due to the weakness of the enzyme present in the polynuclear leucocytes and to the strength of the anti-body which opposes it.

Anti-leucoprotease in the Serum of Birds.—An experiment in which the anti-enzymotic power of pigeon's serum was compared with that of dog's, cat's, and goat's serum showed that the serum of the pigeon failed to prevent active proteolysis caused by dog's leucoprotease. The inhibiting action of hen's serum was further

tested with leucoprotease of dog and was compared with that of dog's serum. The following experiment shows the effect of 0.5 and of 1 cubic centimeter of these sera on twenty milligrams of dog's leucoprotease when allowed to digest coagulated serum under the conditions already described:

	Dog's serum.	Hen's serum.
With 0.5 c.c. serum	9.5 c.c.	14.65 c.c.
With 1 c.c. serum	6.05 c.c.	13.75 c.c.

Twenty milligrams of enzyme in the absence of serum caused digestion of coagulated proteid represented by 16.8 c.c. of 1/10 N. sulphuric acid, the control being 2.1 c.c.

It is evident that the anti-enzymotic action of the serum of both pigeon and of hen for leucoprotease of dog, though appreciable, is slight. Since sera of these birds differ from the mammalian sera examined, it has suggested itself that the enzymes present in the leucocytes of the bird might have peculiarities corresponding to this difference. To obtain inflammatory exudates containing leucocytes, aleuronat was injected into the peritoneal cavity of the hen, but from the resulting exudate a sufficient quantity of cells could not be obtained. Turpentine injected into the subcutaneous tissue caused necrosis and accumulation of a considerable number of leucocytes, but suppuration with softening of the tissue did not occur. A powder prepared by the method previously mentioned from the white fibrinous exudate at the seat of inoculation caused very weak proteolysis, when allowed to act at body temperature upon coagulated proteid in the presence of acid, but failed to digest in the presence of an alkaline reaction. Since previous experiments[23] have shown that the bone marrow of the dog contains that enzyme, namely, leucoprotease, which is characteristic of the polynuclear leucocytes, the proteolytic action of a suspension of cells of the bone marrow from the hen was tested in the presence both of acid and of alkali. The bone marrow removed from the bones was shaken violently in salt solution and forced through a fine sieve; the cells thus obtained were washed several times by centrifugalization and suspended in nine times their volume of salt solution. Five cubic centimeters of this suspension were allowed to act on coagulated proteid at body temperature in

[23] *Loc. cit.*

the presence of 0.2 per cent. acetic acid and of 0.2 per cent. sodium carbonate, and with the reaction of the medium unchanged. The experiments of Hedin and of one of us have shown that while the spleen of various mammals undergoes more active autolysis and digests foreign proteid more energetically in an acid medium, it contains at the same time no inconsiderable quantity of an enzyme which digests in the presence of alkali. The proteolytic action of a suspension of splenic cells from the hen was compared with the similarly prepared suspension of bone marrow from the same bird; for further comparison, a suspension prepared from the liver was used. In the following table the digestion of coagulated proteid produced by these suspensions is represented by centimeters of 1/10 N. acid.

	Bone marrow.	Spleen.	Liver.
With reaction unchanged	3.0 c.c.	3.45 c.c.	2.55 c.c.
With 0.2 per cent. acetic acid	10.7 c.c.	12.15 c.c.	8.6 c.c.
With 0.2 per cent. sodium carbonate	2.85 c.c.	3.05 c.c.	2.35 c.c.
Control	2.15 c.c.		1.8 c.c.

These figures show that the bone marrow of the hen, unlike that of the dog, fails almost completely to cause digestion of proteid in the presence of an approximately neutral or alkaline reaction, but causes active proteolysis in an acid medium. An enzyme similar to leucoprotease of the dog, if present, occurs only in very small quantity. The spleen likewise causes very trivial digestion in an alkaline or neutral medium, but is active in an acid medium; the control for the spleen which is lacking doubtless closely approximates that of the bone marrow. The liver which in an acid medium causes less digestion than bone marrow or spleen almost completely fails to digest in a neutral or alkaline medium. An enzyme which digests in an alkaline medium, and occurs in abundance in the organs of mammals is according to the foregoing observations almost wholly wanting in the tissues, notably in the blood-forming organs of the bird.

The absence in the blood-forming organs of the hen of this enzyme is apparently due to its absence in the polynuclear leucocytes. By injecting turpentine into the peritoneal cavity of the hen, a sterile inflammation results: in two experiments at the end of three

and of four days the cavity was found to contain yellowish fluid, but the intestines are loosely matted together by a white or pale greenish, gelatinous material in which are numerous opaque yellow spots. This exudate consists of fibrin holding serum in its meshes; the characteristic polynuclear leucocytes of the bird are present in large number. Bits of this fibrinous exudate washed with salt solution as free as possible of serum were suspended in acid, neutral, and alkaline solutions of which the volume was brought to five cubic centimeters by addition of .85 per cent. salt solution. After digestion at 37° C. during seven days the result of the experiment was as follows:

	Condition of fibrin.
With 0.2 per cent. acetic acid.	Fine powdery sediment.
With 0.1 per cent. acetic acid.	Much eroded.
In normal salt solution.	Unchanged.
With 0.1 per cent. sodium carbonate.	Unchanged.
With 0.2 per cent. sodium carbonate.	Unchanged.

While the experiment does not demonstrate with certainty that solution of fibrin in the presence of acid is due to enzymotic action, the result agrees with that obtained with bone marrow and demonstrates that autolysis of fibrinous exudate containing polynuclear leucocytes fails to occur in a neutral or alkaline medium. The fibrinous exudate obtained several days after injecting turpentine into the pleural cavity of the dog when washed free from serum undergoes complete autolysis, if immersed in 0.2 per cent. sodium carbonate and kept at body temperature.

CONCLUSIONS.

The inhibiting action of the blood serum upon the enzyme of the polynuclear leucocytes, leucoprotease, is exerted by the albumin fraction of the serum. The albumin fraction contains no proteolytic enzymes.

The globulin fraction of the serum contains no anti-enzyme for leucoprotease; it contains, on the contrary, an enzyme which digests proteids in a neutral or alkaline medium. This enzyme resembles leucoprotease which is present in the polynuclear leucocytes of an inflammatory exudate and in the bone marrow from which these cells are derived, and is doubtless identical with the similar enzyme

occurring in smaller quantity in the spleen. This enzyme which is present in the blood serum is held in check by its anti-enzyme, but the latter is in such excess that the serum as a whole is capable of checking the action of leucoprotease when added in considerable quantity.

Leucoprotease of one mammalian species is inhibited by sera of other mammalian species, but the anti-enzymotic activity of various sera differs; the anti-enzyme of the rabbit's serum is stronger than that of dog's serum, when tested either with dog's or with rabbit's leucoprotease. The co-existence in the rabbit of leucoprotease with feeble strength and anti-body of great activity may explain the absence in these animals of typical suppuration with liquefaction of tissues.

The serum of birds which have been tested, namely, pigeon and hen, almost completely fails to inhibit mammalian leucoprotease (of dog). The polynuclear leucocytes, the bone marrow and the spleen of the hen do not contain an enzyme resembling leucoprotease of mammals. The absence of anti-enzyme in the serum is associated with absence of a corresponding enzyme in the leucocytes.

ON THE ELECTRICAL CHARGE OF THE NATIVE PROTEINS AND THE AGGLUTININS.

By CYRUS W. FIELD[1] AND OSCAR TEAGUE.

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In a previous paper[2] it was shown that the particles of both toxin and antitoxin wandered under the influence of an electric current toward the cathode and that the reaction (acidity or alkalinity) of the solvent did not influence the direction of migration. Since Hardy[3] and Pauli[4] demonstrated that the proteins which they used were amphoteric, *i. e.,* that they pass toward the anode in an alkaline medium and to the cathode in an acid one, there has been a tendency to generalize by assuming that all proteins behave in this manner. If such were the case, we pointed out, it would follow from our experiments that toxin and antitoxin are not true proteins. At the same time, however, we mentioned that from the few experiments in which this question had been considered, the protein matter of the broth or serum seemed in every instance to travel with the toxin or antitoxin toward the cathode. Further experiments have confirmed this result. It was also shown that the protein of normal horse serum and of non-toxic broth travels toward the cathode. Hence our work offers as yet no evidence either for or against the view that toxin and antitoxin are non-protein in nature.

We maintain that the results which Hardy and Pauli obtained, working with denaturalized proteins, are in no wise applicable to the native proteins, but that these carry a distinct electrical charge and are not amphoteric. We are here in accord with Iscovesco[5] and his co-workers, who investigated the charge of colloids con-

[1] Assisted by a grant from the Rockefeller Institute for Medical Research.

[2] Field and Teague, *Journal of Exper. Med.,* 1907, viii, 86.

[3] *Jour. of Physiol.,* 1899, xxiv, 288.

[4] *Hofmeister's Beit.,* 1906, vii, 531.

[5] *Compt. rend. Soc. biol.,* 1906, lxi, 195, 355, 378, 470, 568.

tained in various body fluids. Their method consisted in treating the fluid with electro-negative (arsenic sulphide) and electro-positive (ferric hydrate) inorganic colloids and their conclusions were based upon the fact that colloids of opposite sign when brought together form precipitates. Thus they found that the peritoneal fluid of the horse contains only electro-positive colloids, while the pericardial fluid contains those of both signs; that blood plasma contains both positive and negative albumins with positive and negative globulins, whereas the serum contains only the positive globulin along with albumins of both signs; that the fluid of a tubercular abscess deprived of its leucocytes contains only electro-negative colloids; that the amniotic fluid contains both positive and negative albumins, but only negative globulins. From these experiments Iscovesco concludes that there are no colloids which do not bear a distinct electrostatic charge.

Since our method gave no indication of the presence of an electronegative albumin in normal serum, we are inclined to believe that Iscovesco by his manipulations produced a change in sign of the charge carried by certain proteins and that all of his findings are therefore not applicable to the proteins originally present in the fluids he investigated.

In our previous work with tetanus toxin we investigated only the tetanospasmin and its antibody; we have since shown by testing the agar extracts for their lytic or antilytic action on horse cells that both tetanolysin and antitetanolysin travel toward the cathode under the influence of an electric current. Having determined the electrical charge of toxin and antitoxin, we next applied the same method to an investigation of the agglutinins.

The agar was divided into one centimeter lengths; the agglutinin was found to have traveled seven centimeters into the cathode agar, the anode agar remaining free of agglutinin. The first centimeter length was extracted with five cubic centimeters of water and this extract would still agglutinate at a dilution of 1 : 100.

The specific agglutinins investigated travel toward the cathode. These results are diametrically opposed to those of Biltz, Much and Siebert,[6] who are the only workers, so far as we know, who

[6] *Zeit. für diatet. und phyckal. Ther.*, 1905, viii, 19.

have investigated this subject. They passed a current through lacto-serum contained in a U-shaped tube for from one half to one hour, and found that the fluid around the anode agglutinated at 1:20, that around the cathode not at all, and that from the middle of the U-shaped tube at 1:8. Normally the serum agglutinated at 1:4. They state that after the passage of the current the fluid from around the anode was 1/10 normal acid. We would expect this amount of acid to agglutinate at approximately 1:20, since 1:200 represents about the flocking limit of hydrochloric acid for bacteria.

As stated in a previous article, we took special precautions to eliminate the products of electrolysis. However, to show conclusively that it was the specific agglutinin, and that alone, which was responsible for the agglutination in our experiments, the extracts were also tested against other bacilli than those which were agglutinated by the serum under investigation.

TABLE I.

STRENGTH OF ELECTRIC CURRENT 110 VOLTS; ½ TO 1 MILLI-AMPERE.

Serum agglutinating the typhoid bacillus at 1 : 2000. Current passed for six hours.

Organism.	Cathode Agar cm. Lengths.											Anode Agar.	
	1	2	3	4	5	6	7	8	9	10	11–20	1–10	11–20
B. typhosus.	+++	+++	+++	+++	++	++	+	o	o	o	o	o	o
B. coli.	o	o	o	o	o	o	o	o	o	o	o	o	o
Shiga's bacillus.	o	o	o	o	o	o	o	o	o	o	o	o	o
Para typhoid b.	o	o	o	o	o	o	o	o	o	o	o	o	o
Biuret react.	+	+	+	+	+	+	trace						

NOTE.—As one centimeter lengths of the anode agar showed no agglutinin in repeated experiments we have here tested extracts from ten centimeter lengths.

If the agglutination were due to the presence of products of electrolysis we would expect the other bacilli to be agglutinated as well as typhoid. Such, however, was not the case. Hence, we believe that we have shown conclusively that the agglutinins travel toward the cathode.

It has been shown by Bechhold[7] and Buxton, Schaeffer, and Teague[8] and others that bacteria move toward the anode under

[7] *Zeit. physik. Chem.*, 1904, xlviii, 385.

[8] *Ibid.*, 1906, lvii, 47.

the influence of an electric current, that is, they carry a negative charge.[9] Our findings with regard to the agglutinins is therefore especially interesting, since it shows that in the phenomenon of agglutination we have the combination of an electro-negative suspension with an electro-positive colloidal solution. Since ions of opposite sign are essential for a chemical reaction and colloids of opposite sign when brought together form precipitates, our results harmonize with both the chemical and the colloidal view of the phenomenon.

Bacteria which have been saturated with agglutinin and then washed in a number of changes of water until the wash water contains no more agglutinin were placed in the cell and after eight hours the agar was tested for agglutinin. A small amount was found in the cathode agar showing that under the influence of the electric current the agglutinin bacteria combination was disassociated and that the agglutinins passed to the cathode. Bacteria have been disassociated from agglutinins by other means,[10] but so far as we are aware, this is the first time that disassociation has been affected by means of the electric current.

CONCLUSIONS.

1. Tetanolysin and antitetanolysin travel toward the cathode under the influence of an electric current.
2. The specific agglutinins are electro-positive.
3. The proteid matter of serum is not amphoteric but travels toward the cathode whether its reaction be acid, neutral, or alkaline.
4. The bacteria-agglutinin combination may be disassociated by means of the electric current.

[9] Cernovodeanu and Henri (*Compt. rend. Soc. de Biol.*, 1906, lxi, 200) claim that dysentery bacilli travel toward the cathode but we have not found this to be the case.

[10] Quoted by Eisenberg in *Cent. f. Bakt*, 1906, xxxi, 540 are the following: Joos (if fresh bacilli are added to agglutinated bacilli, which have been previously washed free from serum, the former are agglutinated), Landsteiner and Jagio and Landsteiner and Reich (dissociation at high temperatures).

HETEROTRANSPLANTATION OF BLOOD VESSELS PRESERVED IN COLD STORAGE.

By ALEXIS CARREL.

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For the purposes of the transplantation of blood vessels in man and animal, a method which will suffice to prevent for a time the disintegration which extirpated tissues quickly undergo, would be of great value. With the view of retarding the occurrence of these cadaveric changes, I kept in cold storage for several days blood vessels resected from dogs; and afterwards I transplanted these vessels to cats. It is well known that the tissues of an animal do not grow, or grow hardly at all in an animal of another species. Nevertheless, these heterotransplantations were attempted with the aim of ascertaining whether the vessels, in spite of the toxic action of the cat's blood on the dog's tissue, could adapt themselves to the new conditions of life, and take over the function of the vessels removed. Five experiments were performed.

Experiment I. Female Cat.—A segment of the abdominal aorta located between the mouths of the renal and ovarian arteries was resected. The circulation in the lower part of the aorta was re-established by interposing a segment of the external jugular vein between, and suturing it to the cut ends of the aorta. This segment of the jugular vein had been extirpated from a dog seven days before and kept in an isotonic solution of sodium chloride at or about the freezing point. But the temperature of the refrigerator was not constant, and sometimes varied from 32° F. to 40° F. After the operation was completed, the pulsations of the femoral arteries and of the arteries of the hind limbs were normal. On the second day, the hind limbs became hyperæsthetic. On the third day, paralysis of the hind feet appeared, and a few hours later paralysis of the hind limbs was noted. At the same time, the pulsations of the femoral arteries completely disappeared. On the following days, the paralysis diminished progressively. Twenty-two days after the operation, the animal could walk almost normally. But the pulsations of the femoral arteries did not reappear. By direct examination it was found that the transplanted venous segment was surrounded by a thick layer of vascular connective tissue and its lumen was completely obliterated.

Experiment II. Female Cat.—A segment of the abdominal aorta of a large cat was resected and replaced by a segment of carotid artery which had been extirpated from a young dog twenty days before, and kept in an isotonic sodium chloride solution at about 32° F. Sometimes the temperature of the refrigerator rose for a few hours to 40° F., or even 44° F. After the operation, the pulsations of the femoral arteries and the movements of the hind limbs remained constantly normal. Forty-eight days after the operation, the animal was etherized and its abdomen opened. The pulsations were found normal in the abdominal aorta, and in the segment of carotid. The location of the anastomosis was marked by a slight hardening of the arterial wall. There was little reaction of the connective tissue around the transplanted vessel. The wall of the carotid appeared to be less elastic than that of the aorta. No dilatation of the transplanted segment was observed. The abdomen was closed after the examination, and the animal kept alive. Now, seventy-seven days after the transplantation, the pulsations of the femoral arteries are normal and the animal is in excellent condition.

Experiment III. Male Cat.—A large cat was subjected to an operation similar to the last one. The segment of carotid interposed between the cut ends of the aorta had been extirpated from a dog three days before and kept in dog's defibrinated blood at 32° to 34° F. After the operation, the pulsations of the femoral arteries and the movements of the hind limbs remained constantly normal for thirty-four days. On the thirty-ninth day after the operation it was found that the pulsations of the femoral arteries had disappeared and that both anastomoses were occluded by a small deposit of fibrin. In the lumen of the transplanted segment was a soft clot of recent formation. The thrombosis appears to be due merely to an inclusion and proliferation of the external sheath of the carotid in the line of suture.

Experiment IV. Male Cat.—A small piece of the abdominal aorta of a large cat was resected, and a short segment of carotid artery was interposed between the cut ends. The arterial segment had been extirpated from a dog seventeen days before and kept in defibrinated dog's blood at 32° to 34° F. Ten days after the operation, the pulsations of the femoral arteries became weaker and disappeared completely. By direct examination it was found that the upper anastomosis was obliterated by a small deposit of fibrin. The upper part of the transplanted segment was filled with a clot of recent formation. The lower anastomosis was excellent. The endothelium and the wall of the segment of the carotid were apparently normal.

Experiment V. Male Cat.—An operation similar to the last one was performed on a large cat. The segment of carotid interposed between the cut ends of the aorta had been extirpated from a dog four days before and kept in Locke's solution at 32° to 34° F. After the operation, the animal remained in normal conditions. He was killed six days after the operation. The union of the transplanted segment to the aorta was apparently perfect. There was no deposit of fibrin at the lines of suture and the wall of the segment of carotid artery was apparently normal.

CONCLUSIONS.

The experiments show merely that blood vessels transplanted from dog to cat can act as arteries for seventy-seven days at least; and that having spent several days in cold storage does not interfere with their ordinary functions. The animals operated upon must, however, be kept under observation for several months, or, indeed, for several years, before any conclusion can be drawn concerning the practicability of this method of preservation and heterotransplantation of blood vessels.

NUCLEIN METABOLISM IN A DOG WITH ECK'S FISTULA.

By J. E. SWEET and P. A. LEVENE.

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All nitrogenous substances completely metabolized in the organism of mammals are removed by the urine in form of urea. Every other nitrogenous constituent of the urine is either a substance which has for one reason or another escaped its final transformation, or has not been metabolized by the organism at all. Under metabolism here, of course, is understood a set of chemical reactions which result, first, in maintenance of the integrity of the body's tissue and, second, in supplying it with calories sufficient to perform all the functions of the organism.

The quantity of nitrogen metabolized by the organism for the purpose of energy production is regulated primarily by the intake of nitrogenous food-stuffs, and by the quantity of reserve protein deposited in the organism. The quantity of nitrogen metabolized as a result of tissue deterioration for the purpose of maintaining its integrity is determined by the mass of the cells in the organism, by the amount of work which the tissues are called upon to perform, and by the intensity of the effort employed by the organism in performing the work. Thus, work of short duration, but of such intensity as would occasion a state of fatigue, brings about greater tissue deterioration than prolonged work of lower intensity.

Under normal conditions there exists a definite proportion between the two phases of nitrogenous metabolism, namely, between metabolism of nitrogenous food-stuffs of reserve protein on the one hand, and metabolism of cellular protein on the other. This proportion changes with the change of the plane of nutrition of the organism and with change of the quantity of protein in the food. However, conditions are conceivable in which it is made possible that the organism may be supplied with a sufficient amount of

energy by the sacrifice of the tissue integrity, or conditions may exist in which the tissue integrity is maintained by an unusually high consumption of reserve protein. Thus it is important to be able to trace separately the two phases of nitrogenous metabolism.

Analysis of the intake of food stuffs, a record of heat production, estimation of carbon dioxide and of urea removed by the organism furnish the data required to follow energy production. But one is confronted with considerable difficulty when he attempts to obtain information regarding the condition of the cellular elements of the tissues. This is due to the fact that deteriorated tissue elements are utilized for the purpose of metabolism in the same manner as the protein of the food or as the reserve protein. Their nitrogen is removed in the form of urea. Some components of the cellular elements, however, have a chemical composition distinctly different from that of the reserve protein. These substances, previous to their transformation into urea, undergo a cycle of chemical reactions which is different from that of the reserve protein; the intermediate products of their metabolism differ from those of the latter, and it is very probable that the mechanisms regulating the transformation of the two groups of substances are independent of one another. Indeed, in the light of this assumption one can readily explain the fact that transformation of special components of tissue elements varies considerably in different species, although transformation of simple reserve-protein occurs in an identical manner, and results in the same production of energy.

Among such components of tissue elements special interest is attached to the nucleins, since they constitute the principal substance of the nuclei of all cells. The attempt has been repeatedly made to ascertain the degree of tissue deterioration by analysis of the nuclear derivatives in the urine, the substances of special interest being uric and phosphoric acids. It has been established in recent years that uric acid is formed in the organism through the oxidation of purin bases, which are the principal components of nucleic acids. But uric acid is metabolized further in the organism of mammals, that is, it is oxidized and is finally removed from the organism in the form of urea. Uric acid detected in the urine represents only a fraction of the acid formed in the organism, namely, that part

which has escaped further oxidation. Hence satisfactory information regarding cellular disintegration cannot be gained by the investigation of uric acid output under normal conditions; but the results of uric acid analysis can be made valuable for the solution of the problem, if conditions are found under which the further transformation of uric acid can be prevented.

Clinical observations, and the studies of Pawlow, Nencki, Hahn and Massen especially, have suggested the probability that the liver is the principal organ concerned in the oxidation of uric acid. On the other hand, recent work of Weiner, Burian, Jones and Schittenhelmn have shown that many other animal tissues possess the same power. It must be borne in mind that the Russian investigators were not engaged in the study of purin metabolism especially, and their observations were made at a time when uric acid formation was not yet clearly understood. The facts make a revision of the older work on purin metabolism very desirable.

The present investigation was carried out on a dog in which an anastomosis between the portal vein and the inferior vena cava had been successfully performed by one of us (Sweet). The purposes of investigation were: (1) To ascertain whether or not the output of uric acid was above normal, if the dog was maintaining nitrogenous equilibrium, and was keeping its original weight. (2) To ascertain whether or not the output of uric acid was increased markedly after administration of nuclein or of its derivatives (nucleoproteid of the mammary gland of the cow, nucleic acid of fish sperm, adenin sulphate, and thymin were employed). (3) To ascertain whether the animal possessed the power to metabolize thymin. (4) To ascertain whether or not the output of uric acid was increased on a diet poor in protein material, although containing carbohydrates and fat in quantities sufficient to supply the organism with the required amount of calories. (5) To ascertain whether or not the output of uric acid was increased during starvation.

The present communication represents the results of experiments performed on one dog, and cannot, therefore, serve as a basis for broad generalization. The work is communicated in its present incomplete state, because accidental circumstances render its im-

mediate completion unpracticable; the results already obtained are believed to be of interest.

Methods.—The urine was collected in three-day periods, the dog being catheterized at the beginning and at the end of each period. The receptacle contained a quantity of 10 per cent. solution of sulphuric acid to make up with the urine a solution of about 2 per cent. of sulphuric acid. No other preservative was added.

The nitrogen estimations were made by the Kjeldahl-Gunning method. The phosphorus was estimated gravimetrically, the urine being evaporated and fused with a mixture of potassium carbonate and sodium nitrate. Uric acid was estimated by the Leube-Salkowski method, ammoniacal silver chloride being used instead of the nitrate.

Elimination of Uric Acid.—Information regarding uric acid elimination by dogs with Eck's fistula is limited to the observations of Pawlow, Nencki, Hahn and Massen. Their dogs were fed on a mixed diet, no special attention being paid to the quantity of purin bodies in the food-stuffs, and no special care being exercised in collecting the daily quantities of urine. Moreover, since the dogs used by the Russian investigators developed the typical nervous attacks described by Pawlow, without further evidence the objection might be urged that the high uric acid output was occasioned by deterioration of the nuclear elements of the liver, or was secondary to the convulsive attacks.

In the present work the experiment was not begun until the nutrition of the dog after operation was so much improved that it maintained nitrogenous equilibrium. Furthermore, the dog was fed on cracker dust, plasmon, lard, and salt, to eliminate all purin in the diet, and had been on this diet for several weeks prior to the beginning of our records. It is, therefore, obvious that all the uric acid found in the urine must have originated in disintegration of tissue. The output of uric acid in this experiment was considerably higher than that of a normal dog.

Feeding of Nucleoprotein.—On two successive days the dog received, instead of plasmon, twenty-five grams of nucleoprotein of the mammary gland of the cow. As seen from the table, a slight increase in the uric acid output occurred, but as the nucleoprotein

FEMALE MONGREL SPANIEL—OPERATION, MARCH 5, 1906.

Date.	Dog Received.	Weight.	Total N-Intake.	Total N-Output.	N in Urine.	N in Faeces.	Total Uric Acid.	Injected P_2O_5	P_2O_5 in Faeces.	P_2O_5 in Urine.	Amount of Urine.	Weight of Dry Faeces.
Apr. 27–28–29.	75.0 Cracl	9250	12.891	14.140 g.	12.909	1.231 g.	0.240 g.	3.9045 g.	—	2.826 g.	1500	38.0 g.
30–May 1–2.	75.0 Cracl	9400	"	14.867	12.18	2.687	0.492	"	1.3278 g.	2.254	2020	88.0
3–4–5.	25 g. I dust	9250		14.226	12.78	1.446	0.663		—	2.157	1700	45.0
6–7–8.	Plasmon, Cracker dust, Lard.	9250	"	12.559	11.2	1.359	0.230	"	—	2.113	1000	43.0
*16–17–18.	" + 1 g. Adenin sulphate.	9250		12.118	10.69	2.028	0.620		—	2.317	2190	65.0
19–20–21.	Plasmon, Cracker dust, Lard.	9150	"	14.631	13.8	0.831	1.050	"	—	3.156	3000	43.0
22–23–24.	" + 10 g. Nucleic acid.	—		15.13	13.37	1.766	1.301		2.519	3.947	3420	68.0
25–26–27.	Plasmon, Cracker dust, Lard.	9200	"	13.75	12.53	1.22	0.681	"	—	2.632	2710	58.0
28–29–30.	" " "	—	"	13.804	12.64	1.164	0.721	"	—	2.644	2560	38.0
31–June 1–2.	" + 3 g. Thymin	9200		12.512	11.22	1.292	0.707		—	2.408	3005	45.0
3–4–5.	Plasmon, Cracker dust, Lard.	9300	"	14.106	11.986	2.12	0.644	"	—	2.596	3020	58.0
6–7–8.	" + 6 g. Thymin.	9350		12.874	11.708	1.166	0.755		—	2.940	4425	43.0
9–10–11.	Plasmon, Cracker dust, Lard.	9400	"	12.602	11.401	1.201	0.796	"	—	2.517	2800	38.0
12–13–14.	" " "	9550	"	13.542	11.835	1.617	0.840	"	—	2.690	2720	58.0
15–16–17.	" " "	9650	"	14.068	12.513	1.555	0.797	"	—	2.382	2800	60.0
18–19–20.	" " "	9600	"	14.079	12.699	1.380	0.773	"	0.9322	2.467	2650	48.0
21–22–23.	195 g. Cane sugar, 225 g. Cracker dust, Lard as above.	9700	3.042	9.467	8.808	0.659	0.947	0.774	—	1.846	2800	28.0
24–25–26.	195 g. Cane sugar, 225 g. Cracker dust, Lard as above.	—	"	7.638	7.140	0.498	0.972	"	—	1.622	3260	23.0
27–28–29.	195 g. Cane sugar, 225 g. Cracker dust, Lard as above	10350	"	12.428	8.708	3.720	1.025	"	—	1.902	3590	285.0 †
30–July 1–2.	195 g. Cane sugar, 225 g. Cracker dust, Lard as above.	11200	"	10.824	7.382	3.442	0.857	"	—	1.294	2700	422.0 †
3–4–5.	Starvation.	8700	0.0	7.344	7.344	No faeces.	0.906	None.	0.0	1.518	3200	0.0
6–7–8.	"	8350	0.0	6.65	6.65	No faeces.	0.746	None.	0.0	1.390	1450	0.0

75.0 g. Plasmon (3 days) = 8.835 g. N.; = 2.8725 g. P_2O_5.
300.0 g. Cracker dust (3 days) = 4.056 g. N.; = 1.032 g. P_2O_5.

* May 9–15 not worked out, owing to accidental loss of urine.
† Faeces does not dry thoroughly, owing to large amount of sugar contained.

employed in the experiment had been extracted with alcohol and dried, its absorption and assimilation were perhaps somewhat unsatisfactory. Few experiments in which dogs have been fed on pure nucleoproteins are recorded, but the feeding of organs rich in nucleoproteins and purins in the experiments which have been reported have failed to cause any marked increase in the elimination of uric acid.

Feeding of Adenin.—One gram of adenin sulphate was administered the first day of the experiment. All earlier experiments in which dogs were fed on purin bases failed to show any increase in uric acid output. Only Minkowski[1] records an increased elimination following xanthin or hypoxanthin feeding, but he failed to obtain an increase with adenin. In the present experiment a slight increase in the uric acid output was noticed during the period of feeding; it was more marked in the following period. It is difficult to determine from this one experiment whether the rise was actually occasioned by the administration of adenin or occurred accidentally. Observations are recorded in which increase of uric acid appeared a few days after the administration of nuclear material (Camerer[2]). The increase in uric acid elimination during the period of adenin feeding, and during the following period was 0.3668 gram, an amount equal to 44.27 per cent. of the administered adenin. There was also noted an apparent retention of nitrogen during the period of the experiment, and an increase in the period next following, while during the latter period an increase in the uric acid elimination and diuresis were noted. All these findings may be explained by the action of adenin on the kidneys.

Feeding with Nucleic Acid.—The dog received in addition to its usual food ten grams of the sodium salt of nucleic acid of fish sperm. There was a marked increase of uric acid, an increase of phosphoric acid elimination and increased diuresis. The increase of nitrogen elimination did not exceed 0.8 gram. The nitrogen of this increase apparently had its origin in the absorbed nucleic acid, and corresponded to about 4.8 grams of nucleic acid. In reality, however, part of the increase in nitrogen elimination was occasioned by the

[1] *Arch. f. exper. Path. u. Pharm.*, 1898, xli, 375.

[2] *Zeitsch. f. Biol.*, 1896, xxxiii, 139; 1897, xxxviii, 206.

higher diuresis. According to the analysis of Levene and Mandel,[3] the nucleic acid employed in this experiment contained not more than 0.4 gram of purin bodies. The increase of uric acid elimination was about 0.2000 gram, which represents about 40 per cent. of the purin ingested with the nucleic acid.

In normal dogs an increase in the output of uric acid after nucleic acid feeding is recorded by Minkowski.

Feeding of Thymin.—Feeding experiments with pyrimidin bases have been performed by Stendel[4] with the purpose of studying the synthetic formation of uric acid in the living organism. Administration of thymin remained without influence on the output of uric acid. Stendel's efforts to recover the substance in the urine were unsuccessful.

In the present experiment, the dog received six grams of thymin, which was added to his food. No increase in the total output of nitrogen was noted; there was rather a slight nitrogen retention. On the other hand, a marked diuresis occurred, and the urine contained about 13.5 grams of the ingested thymin.

In order to obtain thymin, one liter of the urine was rendered acid by means of nitric acid and treated for pyrimidin bases by the Kossel-Jones method. The substance obtained in this manner was recrystallized out of very dilute sulphuric acid. A free crystalline base showing all the properties and the appearance of thymin was obtained. The substance had the following composition:

0.1372 grams gave 27.4 c.c. of nitrogen (over 50 per cent. potassium hydroxide) at $t^\circ = 32.0^\circ$ C. and $p = 756$ mm.

For $C_4H_6N_2O_2$:

Calculated $N = 22.22$ per cent.

Found $N = 22.96$ per cent.

Thus the greater part of the ingested thymin is eliminated by the kidneys; but it is difficult to form an opinion as to the extent of the decomposition in the organism, since it is possible that only part of the thymin had been absorbed, while another part may have suffered decomposition in the intestinal tract, through the action of bacteria.

[3] *Zeitsch. f. physiol. Chem.*, 1906, l, 1.

[4] *Ibid.*, 1901, xxxii, 285.

Since it had been observed that the oxidation of thymin in the organism of the dog employed in this experiment was greatly impaired, an attempt was made to discover thymin in the urine obtained during the purin free periods, and also during the periods following the nucleic acid feeding. This was done with a view to establish the extent of nucleic acid decomposition in the organism. It was expected that thymin would be found in the urine, if considerable quantities of it were formed in the organism. From the experience of one of us (Levene[5]) with autolysis of animal organs, and also from that of Jones[6] and of Reh,[7] it is known that on disintegration of tissues, pyrimidin bases are formed. Nevertheless, neither during the period of nuclein free diet nor during that of the nucleic acid feeding, did the urine show any traces of thymin. One is, therefore, led to the conclusion that in the living organism nucleic acid either does not suffer complete disintegration, or disintegrates slowly, the small quantities of thymin thus formed being further decomposed.

Diet with a Low Protein Content.—The object of these experiments was to obtain further information regarding the factors which regulate the output of endogenous uric acid. Our present knowledge of endogenous uric acid is based on a very limited number of observations made on men. On the normal dog the study of nuclein metabolism has not been possible, since the intermediate products of decomposition of these substances are very readily transformed by the animal into urea. However, a dog with Eck's fistula possesses a lower degree of oxidizing power for purin bases than even man. Thus, it is seen, after feeding adenin that nearly 45 per cent. of the substance was eliminated with the urine in form of uric acid. Burian and Schur[8] found in men, after administration of hypoxanthin, a figure approaching this, and one less than half so high after feeding other purin bodies. It is believed by most observers that the output of endogenous uric acid remains practically constant and is totally independent of the quantity and of the quality of the food taken.

[5] *Zeitsch. f. physiol. Chem.*, 1901, xxxii, 546; 1903, xxxvii, 521; 1904, xli, 402.
[6] *Ibid.*, 1904, xlii, 35.
[7] *Hofmeister's Beiträge*, 1903, iii, 569–573.
[8] *Pflüger's Arch.*, 1900, lxxx, 342.

True, Burian and Schur,[9] the principal advocates of this view, considered it necessary to point out that extreme changes in the quantity or in the character of the food may alter the output of endogenous uric acid. Excessive intake of food, which taxes the gastrointestinal glands beyond normal, may result in an increased uric acid elimination, while on the other hand, starvation results in a decreased uric acid output, since it lowers the intensity of general metabolism. Folin[10] much more emphatically expresses the view that the output of uric acid is not invariably the same for an individual, as Burian and Schur[11] claim.

Folin arrives at this conclusion: "When the total amount of protein metabolism is greatly reduced, the absolute quantity of uric acid is diminished, but not nearly in proportion to the diminution in the total nitrogen, and the percentage of uric acid nitrogen in terms of the total nitrogen is, therefore, much increased." L. B. Mendel[12] states, "When the total amount of protein metabolism is greatly reduced, the endogenous output of uric acid is diminished, though this is not the case within ordinary ranges of diet."

A reduction of the intake of albuminous food-stuff should occasion for a time an increased disintegration of the body proteins, and with it a greater destruction of the cellular elements of the tissues. This, in its turn, should result in a greater formation of uric acid. Therefore, it seemed to us probable that the diminished uric acid output, under the conditions indicated by Folin, Mendel, and others, was caused, not by diminished formation, but by more complete combustion of the purin bodies. *A priori*, it might be expected that the organism utilized more completely nitrogenous substances of non-protein nature, when there was a lack of protein in the food. If this assumption were correct, one should find under these conditions an increased elimination of uric acid when the power of the organism to consume purin bodies is diminished. In our experiments, indeed, the diminution of the protein intake and its substitution by fat and carbohydrate (so that the intake of

[9] *Loc. cit.*

[10] *Amer. Jour. of Physiol.*, 1905, xiii, 87.

[11] *Loc. cit.*

[12] Harvey Lecture, *Journal of the American Med. Ass.*, 1906, XLVI, 843, 944.

calories was not altered) was followed by an increase in the uric acid elimination. It was expected that the organism would, on a continued low protein diet, finally adjust itself to the condition and preserve the integrity of the remaining cellular elements. In our dog, the uric acid elimination suffered a marked fall during the fourth low protein period.

Starvation.—In order to understand fully the influence of diet on the output of endogenous uric acid it was considered necessary to ascertain the influence of starvation on uric acid elimination. It was originally planned to have the starvation period precede the period of low protein diet. Since fasting might prove fatal to the dog, it was thought safer to change the order of the experiments. During the first starvation period, the uric acid elimination showed a rise, as compared with the preceding period. The output, however, was not as high as during the early period of low protein diet. Comparing the starvation periods with the periods of diminished protein diet, one gains the impression that the uric acid output was higher during the latter experiment. One must, however, bear in mind that this experiment preceded starvation and the continued low protein diet caused a considerable diminution in the mass of the cellular elements of the body. On the other hand, it is also possible that the absence of activity of the digestive glands is the cause of the lower output of uric acid during starvation.

The Period Following Starvation.—After the second starvation period the animal received the usual diet, consisting of cracker dust, plasmon, and lard. It ate all the food given, but on the next day refused to eat, and later developed the typical symptoms described by Pawlow. Once, typical epileptic convulsions were observed by the writers. The animal remained for forty-eight hours without food, and showed a tendency to recover. It refused the usual food—plasmon, etc.—and also milk, and was then given boiled meat which it ate ravenously, apparently improving. However, after eighteen hours, it again relapsed into the nervous condition described, and was found dead on the morning of the fifth day after the end of the starvation period.

The autopsy showed a condition of extreme emaciation. Aside from cachexia, the organs of the body presented a normal appear-

ance. The liver was possibly of smaller size than usual, but showed no cirrhotic or other lesions. The veins of the abdominal viscera were filled with an injection mass, the vena cava having been tied above the diaphragm, and the site of the anastomosis was minutely dissected; no collateral branches, which sometimes carry portal blood around the ligature about the portal vein, were found. The opening of the fistula was large and perfectly formed.

On the Diuretic Action of Nuclein Derivatives.—A review of the tables clearly shows that a more or less pronounced diuresis follows the administration of nuclear material. The tables of Burian and Schur,[18] and those of Minkowski indicate the occurrence of this phenomenon. In the experiments of these investigators, increased diuresis followed only the administration of nucleins, or nuclein containing tissues, but did not follow the administration of the free purin bases. In our experiments diuresis was less pronounced after the administration of adenin. It is worthy of note, however, that the administration of thymin caused the most pronounced diuresis. As is well known, the methylated dioxypurins possess a much higher diuretic action than other purin derivatives, and it seems probable that the methylated dioxypyrimidin also possesses a high diuretic action. This question, however, can be answered only after further study.

[18] *Loc. cit.*

THE EXPERIMENTAL VASCULAR LESIONS PRODUCED BY BACILLUS MALLEI.

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PLATES VII AND VIII.

In the course of an experimental study of the histological lesions of glanders, the result of which I hope to publish at a later date, I was impressed with the nature and regular occurrence of vascular changes in rabbits and guinea pigs following the injection of *Bacillus mallei* and its poison. The work was not undertaken primarily for the study of the blood vessels, but as it was early observed that the vascular lesions were similar to those produced by the administration of adrenalin and occurring in glanders in man, I considered this part of the histological study sufficiently important to warrant a special communication.

METHODS.

The animals used in this series of experiments were full grown rabbits and guinea pigs. The inoculation was made subcutaneously, intravenously, or intra-peritoneally. The same strain of *Bacillus mallei*[1] was used throughout the work. The original culture was attenuated in virulence by frequent transplantation during a period of eighteen months. Three series of animals were inoculated with definite amounts (1) of an attenuated culture, (2) of a highly virulent culture, and (3) of the cultures killed by heat. The attenuated cultures were obtained as noted above. The highly virulent cultures were obtained by frequent passage of the bacilli through guinea pigs. The killed suspensions were prepared by heating the virulent culture in salt solution to 55° C. The bacilli were prepared for inoculation by growing cultures on slanted

[1] The culture was obtained through the kindness of Dr. Frothingham of Boston.

1 per cent. dextrose-agar for from 24 to 48 hours at a temperature of 37° C.; afterwards washing down the growth and thoroughly emulsifying it in 10 c.c. of normal saline or bouillon. The dosage of the three emulsions was as follows: (*a*) Attenuated culture, 1 c.c. for guinea pigs, 5 c.c. for rabbits. (*b*) Virulent culture, 0.5 c.c. for guinea pigs, 5 c.c. for rabbits. (*c*) Killed culture, 0.5 c.c. for guinea pigs, 1.0 to 1.5 c.c. for rabbits.

Although these were the usual doses employed, another dose of the attenuated bacilli was found useful for the production of a partial immunity in guinea pigs which was to prepare them for larger doses of the virulent culture. These large doses were used to produce certain proliferative vascular changes noted below. The dose in this case was usually 0.5 c.c. of a suspension in which one loopful of a twenty-four hour agar growth had been added to one cubic centimeter of saline. The doses were repeated every third or fourth day over a period of two to four weeks. Rabbits received the injections into the marginal ear vein. Guinea pigs, owing to the smallness of the ear veins, were inoculated into the axillary and femoral veins, aseptic precautions being used. Subcutaneous and intra-peritoneal injections were employed with equally good results. The emulsion of dead bacilli was injected without filtration. The ordinary mallein of the market failed to give results. Occasionally rabbits succumbed to the injection of the living virulent bacilli in from three to five days but this was not the rule; the rabbit is not highly susceptible to the glanders bacillus and easily stands large and repeated doses. Pregnant rabbits and mothers are more susceptible to the inoculations.

Guinea pigs give more constant results than rabbits, although the vascular changes do not differ essentially in the two species. The tolerance to the glanders bacillus was best observed in the female pigs. The difference in susceptibility of rabbits and guinea pigs as regards sex and the bearing of this on glanders immunity has recently been discussed by Nicolle.[2]

For the histological study the tissues were fixed in Zenker's fluid, alcohol or formalin, and embedded in paraffin. Methylene-blue-eosin and phosphotungstic-haemotoxylin were used in the rou-

[2] Nicolle, *Annales de l' Inst. Pasteur,* 1906, xx, 625, 698, 801.

tine staining of sections. Weigert's elastic and Mallory's connective tissue stains were used for the special demonstration of elastic and connective tissues. Sharlach R. was employed tc demonstrate the presence of fat. The methylene-blue-eosin method was more useful as a routine stain than the haemotoxylin-eosin; with the former the early vascular lesions were more readily determined, the calcified areas more distinctly indicated, and the proliferative endothelium and degenerative medial changes were more readily observed. The phosphotungstic-haemotoxylin method was especially useful in marking out the degenerative lesions of the smooth muscle fibres giving to such areas a brownish black color in deep contrast to the faintly stained normal portions of the vessel wall.

The intimal and medial lesions were well differentiated with Mallory's connective tissue stain; indeed this stain alone made possible the separation of the internal elastica of the lesion from the degenerated band of the circular fibres. It stained the internal elastic membrane in the vascular lesion a dark blue in contrast to the normal elastica which was stained a distinct pale blue. The degenerated muscle fibres were stained a deep orange-red in contrast to the purple-blue of the normal muscle cells. The intimal endothelium was stained a salmon color. The medial lesion was stained a deep orange-red owing to some special affinity of the acid fuchsin for degenerating muscle fibres. The deep orange-red of the medial area of degeneration in the immediate proximity to the swollen and straightened dark blue bands of the internal elastica gave a striking picture.

HISTOLOGICAL LESIONS.

For purposes of clearness I will consider the histological lesions under three headings: (1) the exudative, (2) the proliferative and (3) the degenerative. This grouping of the lesions corresponds closely with the degree of the virulence of the cultures employed. In general it can be stated that a highly virulent culture produces vascular lesions of an acute inflammatory type, while a culture of low virulence produces proliferative changes. The degenerative changes are secondary to the proliferative lesion.

While the glanders poison attacks especially the arterial system, and more especially the smaller arteries of the peripheral circulation, none of the arteries wholly escapes injury.

The selection of the smaller arteries by the bacterial poison in preference to the aorta and its larger branches is a striking feature of the two cases of glanders infection studied in man. In both cases the vascular lesions were found in the terminal arteries of 1 mm. to 2 mm. in diameter; and they were especially numerous in the arteries distributed to the skin, lungs, brain and kidneys. The lesion consisted of circumscribed aneurysmal bulgings of the vessel walls showing proliferative intimal and degenerative medial changes (Plate VII, Fig. 1). The diseased arteries were in no way involved in the general infectious process going on in other tissues.

The acute vascular changes occurring in the rabbits and guinea pigs are usually focal in distribution. The most marked lesions occur in the male pig and the female rabbit. Other lesions than the vascular ones, resulting from the injection of *Bacillus mallei*, occur with less frequency.

In animals the earliest change of the vessel noted consists of swelling of the sub-endothelial tissue spaces of the intima due to œdema, the overlying layer of the endothelium remaining perfectly normal. In still other vessels aggregations of polymorphonuclear leucocytes between the intima and the media occur. Often these collections of polynuclear cells are so densely packed as to form layers extending more than half around the circumference. The sub-endothelial collections of leucocytes at the point of greatest density may attain a depth of six to eight cells; while the overlying endothelium shows no break or appreciable protoplasmic or nuclear change. In a longitudinal section of the vessel the sub-endothelial layer of leucocytes may extend, gradually tapering in the long axis of the vessel, for a distance many times that of the circumference. It would appear that the polynuclear zone extends from a primary focus in the direction of the long axis rather than circumferentially. In these vessels showing polynuclear collections, or sub-intimal abscesses, there is a notable absence of fibrin and other cellular elements. The lumen of the vessels involved is often crowded with polymorphonuclear cells, but I was never able to observe them adherent to or penetrating the intimal endothelium. However, one must assume that the sub-intimal lesion results from a wandering out of the leucocytes, and where this occurs in the larger arteries the media offers a barrier to the leucocytic penetration and consequently causes an accumulation of the cells under the intima at some portion of the vessel wall.

Thrombosis is a frequent lesion especially of the vessels at or near the site of inoculation. The ear vessels of the rabbit invariably undergo thrombosis after injection of *B. mallei* or its poison; and not only the vein into which the

material is introduced, but also the other veins and arteries. The thrombosis takes place gradually, spreading from the primary focus which is the point of entrance of the injecting needle. In some animals the greater part of the ear sloughed off. The lesions follow injection of poison or killed bacteria as well as living bacilli. The mesenteric and omental veins are commonly affected after intra-peritoneal injection.

Diffuse or focal infiltration of the media with eosinophile cells appears in the smaller arteries; in one female rabbit dying of acute glanders four days after intravenous injection the media of the mesenteric arteries contains eosinophiles. These collections are focal and apparently invade the vessel wall from the peri-vascular tissue where eosinophiles are present in moderate numbers. Occasionally the eosinophiles are grouped about the vaso-vasorum; in other instances they are collected in areas of well advanced degeneration of the smooth muscle fibers.

Lymphoid and plasma cell infiltration of the vessel wall is rarely met with; it is noted in the omental vessels of two female guinea-pigs after a subcutaneous inoculation.

A peri-capillary lymphoid infiltration is quite common in vessels at or near the site of inoculation.

The proliferative changes are confined exclusively to the intima of the vessels, and are undoubtedly the chief factor in the production of the degenerative changes of the media. The proliferation takes place in the endothelial cell layer and not in the sub-endothelial connective tissue. The degree and extent of intimal reaction depend upon the virulence and dosage of the culture. All grades are seen from a simple heaping up of the cells at one focus, or at many foci, to complete occlusion of the lumen. The proliferated endothelial cells are often phagocytic, containing in their protoplasm red blood cells, altered blood pigment, and fragmented nuclei. This proliferation of the intima is best obtained in the rabbit. In some arteries the phagocytic cells project into the lumen in irregular finger-like masses. Mytotic figures in the attached endothelial cells are often observed. (Plate VIII, Fig. 4.)

In vessels in which a gradual occlusion of the lumen occurs the proliferated endothelial cells undergo degeneration. As the narrowing of the lumen progresses the changes in the occluding endothelial cells advance; finally there is complete necrosis of the cells as indicated by the loss of nuclei and the more or less homogeneous hyaline appearance of the whole occluding mass. In cross sections of small arteries a characteristic appearance is the focal nature of the endothelial proliferation; some parts of the intima show no change while other parts show an enormous "heaping up" of the endothelium. (Plate VIII, Fig. 4.)

The proliferated cells bordering on the lumen are often enlarged to ten times their normal size; direct action of the poison on these border cells may be assumed from the condition of the underlying endothelial cells, which are less than half the size, and are in a good state of preservation. Many of the attached cells bordering on the lumen contain, in addition to large and small fat droplets, numbers of red blood cells, polymorphonuclear leucocytes and nuclear fragments. Frequently these large proliferated cells become detached and are seen in the lumen in a state of marked fatty or hyaline degeneration. These large degenerated cells have been found occluding the sinusoids of the liver and cause

ultimately focal necrosis of the parenchymal cells. Some of the smaller vessels are completely occluded with large detached and degenerated endothelial cells, giving rise to a more or less indefinite hyaline and fatty mass, which mass is not unlike the focal areas of degenerated phagocytic endothelial cells seen in the Malpighian bodies of the spleen in certain acute infectious diseases. (Plate VII, Fig. 3.) This phagocytic action of the intimal endothelium of the vessels occurs with remarkable frequency in rabbits and guinea-pigs following the intravenous injection of a glanders culture of moderate virulence. In addition to the phagocytic property of the intimal endothelium there is a still more striking feature in the occurrence of giant cells, which appear as perfectly formed multinucleated elements having a well defined protoplasmic limitation. (Plate VIII, Fig. 6.) These giant cells may be seen attached along the outermost layer of the endothelial heaps. They contain from four to seven nuclei which are usually situated excentrically. There is no doubt as to the endothelial origin of these elements, a fact of importance in considering the histogenesis of giant cells in general. Still another type of proliferation is that seen in vessels in which the endothelium increases evenly around the whole circumference in the form of concentric lamellæ until there occurs complete obliteration of the lumen. In this form of lesion the endothelium always maintains itself in even layers and at no period, even after occlusion, are the cells found degenerated or in a poor state of preservation as in the case of the obliterating endarteritis resulting from the phagocytic cell occlusion.

In the vessels showing this particular form of obliteration by means of superimposed layers of intimal cells, the musculature and adventitial coats increase proportionately to the intimal increase.

The media attains an enormous thickness, and as there cannot be made out any increase in the number of the nuclei of the media an actual hypertrophy of the smooth muscle fibers probably occurs. The adventitia increases even beyond the thickness of the media. Such vessels now appear as enormously hypertrophied closed tubes, each layer sharply defined and proportionately thickened.

A word may be said about the changes noted in the lymphatics. These vessels are dilated even to many times their normal size. The single lining layer of endothelial cells is swollen and finely granular. In many of these cells the protoplasm is filled with small fat droplets. An occasional large endothelial cell may be found in the lumen together with a large amount of serum. The changes in the lymphatics are especially marked in the omentum and in the abdominal parietes after the subcutaneous and intra-peritoneal injection of *B. Mallei*. Occasionally an endothelial cell lining the lymphatic shows a well-defined mytotic figure.

The degenerative lesions are secondary to the proliferative lesions. They always occur in the innermost layer of the media and in that portion of the vessel wall which shows an intimal heaping up of the endothelial cells. At such points in the media the vessels ranging from 0.5 to 1 mm. in diameter show under the low power of the microscope circumscribed bulgings or sacculations of the wall together with broad bands of degeneration which lie immediately external to the internal elastic membrane. The internal elastic membrane is between the sub-endothelial layer on the inner side and the degenerative lesion

of the media on the outer side. There is absolutely no proliferative or degenerative change in the sub-endothelial layer of the intima. In this situation the internal elastic membrane loses its normal sinuous curves, straightens out and becomes a fusiform band with a thickened central fibrillated portion, and the narrower distal portions many times thicker than the normal membrane. Throughout the lesion it has the appearance of dipping deeply down beneath the intima, but this dipping is only apparent and is due to the fact that the endothelium of the intima is proliferated to form several layers of cells. (Plate VII, Fig. 2.) There is an aneurysmal bulging of the vessel wall at the site of the lesion and this bulging outward of the vessel wall is so great that even the circumscribed heaping of endothelial cells fails to fill the increased convexity of the vessel at this point. The section of one of these smaller arteries resembles in contour the section of an eye in its antero-posterior diameter, the sacculation corresponding to the bulging of the cornea.

The degenerative lesion begins in the circular fibers of the innermost layer of the media at a point directly external to the point of greatest thickening of the intima. Its tendency is to extend along the circular muscle fibers maintaining the same thickness throughout; it is only in the advanced lesions that it spreads to the fibers of the central zone. In the advanced lesions cross sections of the larger vessels often show the degenerated medial band extending beyond the primary lesion. The longitudinal sections also show that the medial degeneration in advanced disease of the vessels may extend beyond the primary intimal lesion.

The earliest change in the smooth muscle fibers occurs in the form of fatty granules grouped about the nuclei. Later these fat particles increase in number and in time coalesce and form large globular masses. The nuclei become indistinct and finally disappear. The fibers undergo rapid degeneration and ultimately calcification. (Plate VIII, Fig. 5.) This medial change may occur at first in a single isolated smooth muscle fiber, or in a small group of fibers immediately external to the elastica interna. This selection of single cells by the glanders poison is comparable to the action of certain toxins which give rise to necrosis of single cells or small groups of cells in the liver.

The degenerative changes in the internal elastic lamina are similar in nature to those occurring in the muscle fibers and occur simultaneously with them.

In no case was there a degenerative process in the media without proliferation of the intima; but proliferation of the intima frequently occurred when there was no change in the media.

DISCUSSION.

The vascular lesions in experimental glanders afford an excellent opportunity to study the early intimal and medial changes in experimental arterio-sclerosis. In addition to the degenerative changes analogous to those produced by adrenalin, the bacillus and its toxin produce lesions of an exudative and proliferative character. The kind and severity of the inflammatory conditions would seem to depend largely upon the virulence of the culture.

Various endarterial lesions, for the most part of a degenerative character, have been described by different workers, but the forms produced by the glanders toxin, especially the inflammatory ones, receive no mention. Some authors have regarded the experimental lesions produced with bacteria as analogous to arterio-sclerosis in man, but this view is not generally accepted, and has been disputed by the investigators of adrenalin arterio-sclerosis.

Gilbert and Lyons[3] were the first to produce experimental aortic lesions by the injection of bacteria and their toxins (1) with, and (2) without mechanical injury to the vessel wall. They speak of the successive giving away of the smooth muscle cells, new formation of connective tissue, and calcification of the elastic fibres. In their experiments the aorta alone showed change, which fact together with the sclero-calcareous nature of the lesions lead them to regard the lesions as analogous to aortic sclerosis in man.

Crocq[4] found it necessary to injure the aortic wall in order to produce arterial lesions by bacterial injections. He employed diphtheria, typhoid, and colon bacilli and streptococci in his experiments. Pernice[5] was unable to obtain with the diphtheria bacillus and the staphylococcus lesions in the aorta without first injuring the wall by mechanical means. He especially emphasized the occurrence of a "round cell" infiltration about the vaso-vasorum. Thérèse[6] experimented with bacterial products and describes "round cell" infiltrations of the peri-vascular tissue and capillaries which he attributed to the effect of toxins.

Although changes in the intima and media have been produced separately and described by numerous experimenters with bacteria, no mention appears in the literature, so far as I can find, of medial changes following and dependent upon primary intimal lesions, which occur with such frequency in the vessels of experimental glanders. Workers with bacteria in the experimental production of arterio-sclerosis describe lesions occurring in the aorta but do not mention peripheral vascular changes. It is now well

[3] Gilbert and Lyons, *Archiv de med. exper.*, 1904, xvi, 13; *Compt. rend. Soc. de Biol.*, 1889, xli, 583.

[4] Crocq, *Arch. de med. exper.*, 1894, vi, 583.

[5] Pernice, *Atti del R. acad. delle Scienze med. in Palermo*, 1895.

[6] Therese, Thesis, Paris, 1893.

recognized that in man sclerosis of the peripheral vessels is not infrequently found when there is no demonstrable lesion in the aorta. Moenkeberg[7] described calcareous lesions spreading distally in the vessels of the human extremities while the aorta and its main branches remained unaffected.

The injection of cultures of *Bacillus mallei* of low virulence almost invariably gives rise to lesions in the smaller arteries, while the aorta and its larger branches remain free even from microscopic changes. On the other hand a highly toxic killed culture produces aortic as well as peripheral lesions. The repeated administration of the autolyzed product of highly virulent glanders bacilli produces marked proliferative and degenerative changes in the walls of the aorta and the smaller vessels. These facts would seem to indicate that the aorta and its larger branches are better able to resist this bacterial poison than the peripheral arteries.

Erb[8] states that the adrenalin lesions often occur in the arteries supplying the various organs, and draws attention to those in the renal arteries.

The vascular lesions in the two cases of fatal glanders infection in man were of the sub-acute type, the patients having died of a glanders septicaemia one after six and the other after eight weeks of illness. In these two cases of human glanders, the disease manifested itself as a bronchopneumonia followed by a papular and pustular skin eruption.

In them the aorta and its larger branches were remarkably free from visible sclerosis, showing only a few scattered yellow streaks along the arch and in the abdominal aorta just before its bifurcation. The remarkable features of both cases are the focal vascular lesions of the smallest arteries, especially those of the lungs, muscles and skin. These vascular lesions are by no means confined to the areas of inflammation but are found throughout the peripheral arterial system.

Microscopically the earliest lesions show a focal heaping of one portion of the intimal endothelium. Immediately external to this intimal proliferation the internal elastic membrane becomes straight-

[7] Moenkeberg, *Virchow's Archiv*, 1903, xi, 141.

[8] Erb, *Archiv fur exper. Path.*, 1905, liii, 173.

ened and somewhat swollen. Associated with the intimal heaping there is often a narrow band of degeneration of the innermost layer of the media which lies in close proximity to the intima and separated from it only by the swollen internal elastic membrane.

In the experimental animals, as well as in the human cases, the microscopic study of the peripheral vessels reveal focal changes in the smaller arteries of the internal organs. In general the lesions consists of a circumscribed area of proliferation or "heaping up" of the intima with a corresponding well defined band of degeneration in the smooth muscle fibres of the media. This area of degeneration is always situated in both human and experimental disease in the "inner portion" of the media.

Almost without exception the workers with adrenalin describe the medial changes in the aorta of rabbits as situated in the "central zone" of the middle coat (Josue, Fischer, Pearce and Stanton, Lissauer, Klotz).[9] They explain the difference in situation of the changes in man and in rabbits by the difference of the aorta in man and in rabbits. The intima of the human aorta is a much more complicated structure than the intima of the rabbit's aorta. It contains the so-called musculo-elastic layer. The differences, however, between the smaller arteries of man and of the rabbit and guinea pig are so small that they may be disregarded. The glanders poison produces identical lesions in the peripheral vessels of man, the rabbit and the guinea pig.

Great stress has been laid upon the extensive proliferation of the intima in human arterio-sclerosis which is said to be absent in experimental adrenalin lesions in rabbits. The vascular changes produced by the injection of adrenalin into rabbits have been entirely of a degenerative character and have been described as occurring in the "middle zone" of the media. Adrenalin it seems rarely if ever gives rise to any proliferative change in the intima. The glanders lesion, on the other hand, is caused by primary proliferative changes in the intima of the peripheral vessels, followed by secondary degenerative changes in the media.

[9] Fischer, *Deutsche med. Woch.*, 1905, xxxi, 1713. Pearce and Stanton, *Jour. Exper. Med.*, 1905, viii, 74. Lissauer, *Berlin. klin. Woch.*, 1905, xlii, 675. Klotz, *Jour. Exper. Med.*, 1905, vii, 633; 1906, viii, 504; 1906, viii, 322.

Of the many workers who have approached the question of arterio-sclerosis with experiments on animals, one group has confined their efforts to the injection of bacteria and their toxins with or without first injuring the inner wall of the vessel; another group has injected substances such as nicotine (Adler and Hensel)[10] and adrenalin. The results obtained by both groups of observers have attracted much attention but they have been so varied as to leave us still in doubt as to the origin of arterio-sclerosis. A number of workers have produced proliferative intimal lesions in the aorta by the injection of certain bacteria and their toxins, or lesions of a purely degenerative nature in the media; but nowhere can I find both these types of lesions described as the result of the action of any one species of bacterium and its toxin.

In the vascular lesions produced experimentally by the injection of the glanders bacillus and its toxin we have intimal lesions associated with changes in the media analogous to similar combined changes produced by this bacillus or its poison in the blood vessels of man. Not only are the intimal and medial changes associated but they occur in such a manner as to leave no doubt that the intimal lesion is primary and that the medial lesion is secondary. The degeneration of the media is the result of the proliferative intimal change. This first evidence of degeneration in the experimental lesions and in the human lesions occurs in the "innermost zone" of the media.

CONCLUSIONS.

1. *Bacillus mallei* and its poison produce a variety of vascular lesions in the rabbit and the guinea pig.

2. The type of the lesion depends upon, (*a*) the virulence of the culture, (*b*) the sex of the animal and (*c*) the degree of acquired immunity.

3. The vascular changes of a proliferative and degenerative nature produced by the slow action of the glanders poison in rabbits and guinea pigs are analogous to the vascular lesions caused by sub-acute glanders infection in man.

4. The most common site of the glanders vascular lesions of animals and man is the peripheral vessels, and especially the smaller visceral arteries.

[10] Adler and Hensel, *Jour. Med. Research,* 1906, xv, 229.

5. The aorta is a less common site of the experimental lesions.

6. The vascular lesions produced experimentally by *Bacillus mallei* and its poison consist of three processes, (*a*) exudation, (*b*) proliferation, (*c*) degeneration.

7. The lesions produced by sub-acute glanders in man consist of two processes, proliferation and degeneration.

8. The primary reaction of the vessels in experimental animals and in sub-acute human glanders consists of a proliferation of the endothelium of the intima.

9. The first degenerative changes observed in experimental animals and in sub-acute human glanders occur in the "innermost layer" of the media and not in the so-called "middle zone."

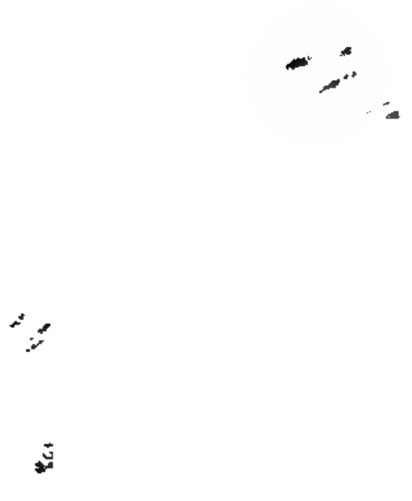
10. The cause of the degenerative change in the inner layer of the media appears to be interference with the nourishment of the circular muscle fibres of the media by proliferation of the endothelium of the intima.

FIG. 1.

FIG. 2.

FIG. 3.

Fig. 4.



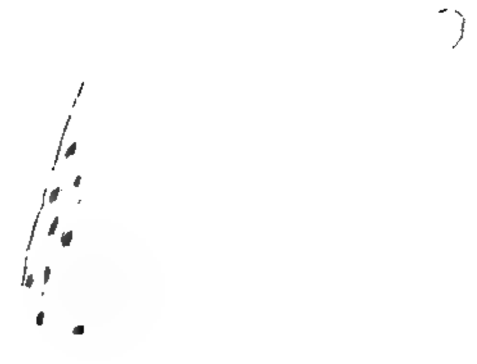


Fig. 5.

Fig. 6.

EXPLANATION OF PLATES VII AND VIII.

FIG. 1. Cross-section of human artery 2 mm. in diameter, from a case of fatal glanders infection, stained after Mallory's connective tissue method. There is to be noted a definite circumscribed bulging of the vessel wall, in which the lesion consists (1) in the "heaping up" of the intima, (2) the straightened and swollen elastica, and (3) the "innermost" layer of medial fibers in a marked state of degeneration. Degenerated fibers are stained a deep orange-red and are in close proximity to the diseased internal elastica.

FIG. 2. Cross-section of a rabbit's artery 2 mm. in diameter stained with methylene-blue-eosin, showing lesion following the repeated intravenous injection of *B. mallei.* Note the zigzag calcified degeneration of the internal elastica and the innermost fibers of the media. The lesion is immediately external to and associated with the intimal proliferation.

FIG. 3. Cross-section of a small artery of a rabbit stained with methylene-blue-eosin, which shows almost complete obliteration of the lumen with the proliferated intimal endothelium. Many of the innermost cells are seen as detached phagocytes undergoing fatty and other degeneration changes. The innermost proliferated cells of the endothelium are compressed into layers and show good preservation.

FIG. 4. Section of a small artery of the rabbit stained with methylene-blue-eosin. Note the enormous size of the proliferated endothelial cells, which are phagocytic and undergoing fatty degeneration. Also note the attached endothelial cell undergoing mitosis.

FIG. 5. Small artery of the guinea-pig showing (1) marked vacuolation of the "heaped up" intimal endothelium, (2) calcified degeneration of groups of smooth muscle fibers of the innermost portion of the media.

Fig. 6. Small artery of the guinea-pig stained with methylene-blue-eosin. Section shows focal proliferation of the intimal endothelium with the formation of "giant cells." Also shows degeneration and calcification of the internal elastica and innermost fibers of the media.

ELECTRIC CHARGES CARRIED BY THE HEMOLYSINS.

By OSCAR TEAGUE and B. H. BUXTON.

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Field and Teague[1] have recently shown that toxins and antitoxins under the influence of the electric current migrate toward the cathode, and in a second series of experiments they found that the agglutinins of typhoid immune serum also travel toward the cathode.

With hemolysins the conditions are somewhat more complicated than with toxins or agglutinins owing to the presence of two factors, amboceptor and complement, being necessary to complete the hemolytic system. It has been shown by Lillie[2] and Henri[3] that red corpuscles migrate toward the anode in an electric current. Provided, therefore, that neutralization of electric charges is a factor in the combinations necessary to effect hemolysis, one would suppose that the immune bodies should travel towards the cathode and the complement toward the anode. We have indeed determined that hemolytic immune bodies migrate in the direction of the cathode, but our experiments indicate very strongly that complement also travels in the same direction.

The immune serums were procured by inoculation into rabbits, of ox and horse corpuscles, washed free of serum, and suspended in an equivalent amount of salt solution, the blood being drawn from the ear or the carotid after intraperitoneal injection of thirty to forty cubic centimeters of the corpuscles in individual doses of five to six cubic centimeters. The immune serums are designated as rabbit v. horse and rabbit v. ox. For complement fresh normal rabbit serum has been used throughout. The accompanying tables refer, some to rabbit v. horse, and some to rabbit v. ox serum, but

[1] *Jour. of Exp. Med.*, 1907, ix, 86.

[2] *Am. Jour. Phys.*, 1903, viii, 273.

[3] *Compt. rend. Soc. de Biol.*, 1904, lvi, 867.

the results with both kinds of serum have been practically identical. There have been slight variations from one experiment to another, but variations of degree only, not of kind, and the tables have been selected from the more typical experiments.

The first cell used was similar to that devised by Field and Teague for their experiments with toxins and antitoxins, and is

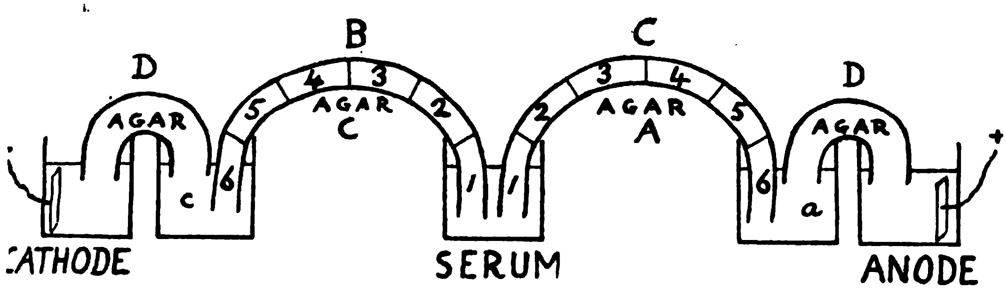

Fig. 1.

shown in Fig. 1. But instead of the platinum electrodes used by them we substituted copper and zinc, so that by surrounding the copper electrode (cathode) with copper sulphate and weighing it before and after the experiment we were able to determine the average current passing through the cells. By surrounding the zinc electrode with zinc sulphate, polarization was avoided, and a more uniform current obtained. The ordinary lighting circuit of 110 volts was used throughout. In Fig. 1 the semicircular tubes *A, C, D, D,* contain one per cent. agar made up with physiological salt solution, and salt solution is also used in Cells *a* and *c,* while the center cell contains the serum to be tested. In order to be certain that no change in the reaction of the central parts of the apparatus should occur during the passage of the current the two outer agar tubes, *D, D,* were made slightly alkaline and a trace of phenolphthalein added. It was found that a considerable amount of acid travelled from the anode and quickly decolorized the anode tube, *D,* which was therefore changed several times during the course of each experiment. Repeated tests showed that under these conditions, there was no perceptible change in the reaction of the three central cells, or in the agar tubes *A* and *C.* Our first

plan was to suspend the red corpuscles in the agar of the tubes *A* and *C* and watch their progressive destruction as the current carried the antibodies from the serum through the agar in one direction or the other.

Test.—Sensitized red corpuscles (ox or horse) suspended were in the agar *A, C,* and fresh normal serum (complement) in center cell of Fig. 1. The result was negative. No destruction of red corpuscles occurred in either of the agar tubes. Repeated and varied experiments on these lines always ended in failure. The red corpuscles in the agar tubes never showed any signs of hemolysis.

The next plan adopted was to fill the agar tubes *A* and *C* with plain agar made up with salt solution, and, after passing the current for four or five hours with serum in the center cell, to cut up the agar into pieces indicated by the numbers in Fig. 1. Each piece of agar was then rubbed down in a mortar, extracted over night in the ice chest with four cubic centimeters of salt solution, and the extracts tested next day for their hemolytic properties. This procedure was the same as that adopted by Field and Teague for their experiments with toxins.

In making the tests given in the accompanying tables we have always used a total of 1.5 cubic centimeters of fluid in each test tube composed of:

1. The immune serum,	.5 c.c.
2. The complement serum,	.5 c.c.
3. Five per cent. suspension of the corpuscles.	.5 c.c.

If therefore we find in the tables that complement serum 0.3 dilution has been used, the final dilution in the test tube is 0.1, or one third of the dilution as given in the table. The same applies to the immune serum, the final dilution being always one third of that given in the tables.

Experiments with cell shown in Fig. 1.—Agar extracts.

Table I.—In center cell is inactivated (56° C.) immune serum. Agar extracts are tested next day with complement. The cathode extracts, 1, 2 and 3, hemolyze the red corpuscles, indicating that the immune bodies travelled into the semicircular agar tube, *C,* in the direction of the cathode. The anode extracts, on the other hand, are not hemolytic.

Table II.—In center cell is fresh immune serum, *i. e.,* complement and immune bodies together; the agar extracts are tested next day with red cells. The results are negative. The hemolytic principles of the fresh immune serum do not appear to travel together through the agar.

Table III.—The extracts of Table II are treated with complement. The table shows that the cathode extracts, 1 and 2, contain immune bodies which have travelled towards the cathode, but the complement of the fresh immune serum has not travelled with them, or Table II would have shown positive results at the cathode end.

Table IV.—In center cell is fresh normal serum. Agar extracts are tested next day with inactivated immune serum. The results are negative. There is no indication that the complement of the fresh normal serum has travelled in either direction.

Conclusions with Cell I.—Immune bodies travel toward the cathode, but there is no indication that complement travels in either direction.

Since the results with complement had so far been negative, certain possible reasons for failure were taken into consideration.

1. During the experiments the agar tubes became warmed owing to resistance to the electric current. The heating might be sufficient to destroy or weaken the complement.

2. Complement may be absorbed by the agar.

To meet the first of these possible reasons for failure the cell was modified in such a way that the serum and the agar tubes could be packed in ice during the course of the experiment. Fig. 2 shows this form of cell, the principle being the same as with Cell 1, but with the serum in a closed tube attached by rubber joints, *J J*, to the semicircular agar tubes.

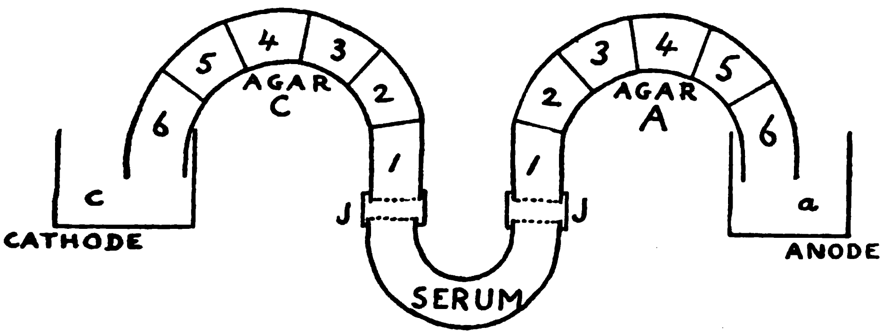

Fig. 2.

With this cell immune bodies could again be shown to migrate readily towards the cathode, but, as with the first cell, tests with complement were always negative. No complement could ever be demonstrated in the agar extracts. It has seemed probable, therefore, that the complement is absorbed by agar.

It has for some time been well established that complement is absorbed and neutralized by organ cells, bacteria, yeast, specific precipitates, and similar substances even though the complement may have no apparent action upon the agents of its absorption. Among the more recent experiments are those of Landsteiner and Stankovich,[4] who demonstrated absorption of complement by various inert substances in suspension or in colloidal solution, kaolin, casein, glycogen, and certain lipoids, such as cholesterin. Table V shows that agar also has this neutralizing action on complement. Fresh normal serum, after treatment with agar has no complementary action on immune serum, whereas the same serum merely filtered through gauze is quite active.

To eliminate the absorption of complement by agar several other cells were devised and tested, but the only one which afforded satisfactory rsults (Fig. 3) consists of a glass tube divided into three compartments by two stopcocks. The whole of the tube is filled with the serum to be tested, and the two outer chambers are connected by a rubber joint, *J*, with curved agar filled tubes; the distal ends of the curved tubes being dipped into cells containing salt solution and corresponding to Cells *a* and *c* in Fig. 1. While attaching the curved agar tube to the cell it is necessary to stick a small capillary glass tube into the rubber to allow some of the serum to escape. If this precaution is not taken the increased pressure will force the agar out of the curved tube. Once the

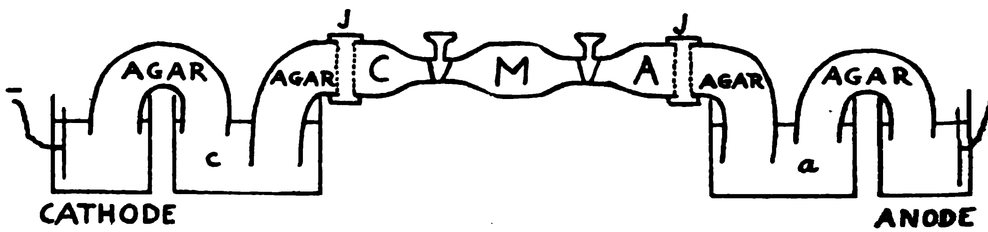

Fig. 3.

agar tube is in position the capillary can be taken out and the minute hole in the rubber closes up of itself. During the passage of the current the cell is packed in ice to prevent heating.

[4] Cent. für Bakt., 1906, xlii, 353.

After the current has passed for four or five hours the stopcocks are closed, and the contents of each chamber *A, C, M,* collected separately and tested for complement content.

Table VI shows that on addition of immune serum the fluid from the cathode chamber, *C,* is distinctly more hemolytic than the fluid from the anode chamber, *A,* while the fluid from the central chamber *M,* holds an intermediate position. It appears from the table that the *C* fluid is always just one dilution ahead of the *A* fluid, so we may conclude that it contains approximately twice as much complement. It is probable, therefore, that complement has a tendency to migrate towards the cathode, and may consequently be regarded as carrying a positive charge.

There is, however, the possibility that the amboceptors of normal serum form a loose combination with complement, so that the complement might be dragged along in the current by the amboceptors, itself remaining passive. But the balance of evidence is decidedly against the probability of any such combination, and, without discussing the subject in detail we may simply say we do believe that it occurs with either hemolytic or bacteriolytic amboceptors.

In the course of a number of experiments with this cell the chamber, *C,* has always contained approximately twice as much complement as the anode chamber, *A,* although occasionally the center cell, *M,* shows as much as, or even slightly more complement than *C.* We are inclined to attribute this occasional slight excess in *M* to some absorption of the complement in *C* by the agar with which it is in contact.

As a control for the experiments with complement serum we have also made some tests with immune serum in Cell III, as shown in Table VII. We find from this table that, on addition of complement, the fluid in chamber *C* is approximately twice as hemolytic as that in chamber *A.* This corresponds very closely with the relative strength of the fluids observed in the experiments with complement of normal serum.

Since we already know that immune bodies travel towards the cathode, and under the conditions afforded by Cell III, do not migrate more readily than complement, we may regard the experi-

ment as going far towards confirming the view that complement also tends to travel in the direction of the cathode.

Hemagglutinins.—Field and Teague[5] have shown that typhoid agglutinins migrate towards the cathode, and in the course of our experiments with hemolysins we have been able to demonstrate the same for hemagglutinins, as shown in Table VIII. The cathode extracts agglutinate red corpuscles but the anode extracts do not.

Summarizing all experiments so far as they have gone, we may observe that Field and Teague have shown that toxins and antitoxins (diphtheria and tetanus) tetanolysin, antitetanolysin, and typhoid agglutinin travel towards the cathode. The experiments detailed here show that hemolytic amboceptors, hemagglutinins, and probably also complement travel towards the cathode. Apparently, then, all the active principles of serum concerned in antireactions have a tendency to migrate towards the cathode.

TABLE I.

Serum rabbit v. ox (56° C.) undiluted. Hemolytic value 0.001.
Current for five hours. Amperes, 0.036.

Extracts.—Agar tubes divided into six pieces. Each piece extracted with four cubic centimeters of salt solution, kept on ice overnight, and tested next day with complement.

Agar Extract 0.5 c.c.	Complement.	Ox Cells.	Hemolysis in	
			3 Hours.	24 Hours.
Anode extract 1.	0.3	0.5 c.c.	—	—
" " 2.	0.3	0.5	—	—
" " 3.	0.3	0.5	—	—
" " 4.	0.3	0.5	—	—
Cathode extract 1.	0.3	0.5	+++	+++
" " 2.	0.3	0.5	+++	+++
" " 3.	0.3	0.5	+	++
" " 4.	0.3	0.5	—	—
Salt solution.	0.3	0.5	—	—

TABLE II.

Fresh immune serum rabbit v. ox undiluted. Hemolytic value .001.
Current for five hours. Amperes, 0.034.
In each tube: Agar extract, 0.5 c.c.; salt solution, 0.5 c.c.; ox cells, 0.5 c.c.

Agar Extract 0.5 c.c.	Hemolysis in 24 Hours.	Agar Extract 0.5 c.c.	Hemolysis in 24 Hours.
Anode extract 1.	—	Cathode extract 1.	—
" " 2.	—	" " 2.	—
" " 3.	—	" " 3.	—
" " 4.	—	" " 4.	—

[5] *Jour. of Exper. Med.*, 1907, ix, 222.

Table III.

Serum and current as in Table II.

In each tube: Agar extract, 0.5 c.c.; complement serum at 1/10 dilution, 0.5 c.c.; ox cells, 0.5 c.c.

Agar Extract 0.5 c.c.	Hemolysis in 1 Hour.	Hemolysis in 24 Hours.	Agar Extract 0.5 c.c.	Hemolysis in 1 Hour.	Hemolysis in 24 Hours.
Anode extract 1.	—	—	Cathode extract 1.	+++	+++
" " 2.	—	—	" " 2.	+++	+++
" " 3.	—	—	" " 3.	—	—
" " 4.	—	—	" " 4.	—	—

Table IV.

Fresh normal serum, undiluted.

Current four hours. Amperes 0.035.

In each tube: Agar extract, 0.5 c.c.; immune serum rabbit v. ox at 1/10 dilution, 0.5 c.c.; ox cells, 0.5 c.c.

Agar Extract 0.5 c.c.	Hemolysis in 24 Hours.	Agar Extract 0.5 c.c.	Hemolysis in 24 Hours.
Anode extract 1.	—	Cathode extract 1.	—
" " 2.	—	" " 2.	—
" " 3.	—	" " 3.	—
" " 4.	—	" " 4.	—

Table V.

Normal serum 1/10 dilution, mixed with pieces of crushed agar, and allowed to stand overnight in ice chest. Next morning filtered through gauze.

In each tube: Filtrate, 0.5 c.c.; rabbit v. horse serum (1/10 dilution), 0.5 c.c.; horse cells, 0.5 c.c.

	Dilution.	Hemolysis in 1 Hour.	Hemolysis in 3 Hours.		Dilution.	Hemolysis in 1 Hour.	Hemolysis in 3 Hours.
Serum 0.2 dilution filtered through gauze.	1/1	++	+++	Serum 0.2 dilution+agar and filtered through gauze.	1/1	—	—
Ditto.	1/2	Trace.	+	Ditto.	1/2	—	—
Ditto.	1/4	—	—	Ditto.	1/4	—	—

Fresh normal serum after treatment with agar has no complementary action on immune serum, whereas the same serum merely filtered through gauze is active.

TABLE VI.

Stopcock cell, Fig. III, containing normal serum, $\frac{1}{10}$ dilution
Current for four hours. Amperes, 0.02.

	Fluid from Compartments.	Dilution of Fluid.	Immune Serum R. v. Horse.	Horse Cells.	30 min.	1 Hour.	4 Hours.
1	Anode A.	1/1	.1	0.5	+	+++	+++
2	"	1/2	.1	0.5	—	+	++
3	"	1/4	.1	0.5	—	—	Trace.
4	"	1/8	.1	0.5	—	—	—
5	Cathode C.	1/1	.1	0.5	+++	+++	+++
6	"	1/2	.1	0.5	+	+++	+++
7	"	1/4	.1	0.5	—	+	++
8	"	1/8	.1	0.5	—	—	Trace.
9	Center M.	1/1	.1	0.5	++	+++	+++
10	"	1/2	.1	0.5	—	++	+++
11	"	1/4	.1	0.5	—	—	+
12	"	1/8	.1	0.5	—	—	—
13	Salt sol.		.1	0.5	—	—	—
14	Salt sol.		Salt sol.	0.5	—	—	—

TABLE VII.

Stopcock cell, Fig. III, containing immune serum, rabbit v. ox, dilution $\frac{1}{10}$.
Current four and one half hours. Amperes, .02.

	Fluid from Chambers 0.5 c.c.	Dilution of Fluid.	Normal Serum 0.5 c.c.	Ox Cells.	Hemolysis in		
					1 Hour, 30 Min.	2 Hours.	3 Hours.
1	Anode A	1/1	0.075	0.5	+++	+++	+++
2	"	1/2	0.075	0.5	+++	+++	+++
3	"	1/4	0.075	0.5	++	++	+++
4	"	1/8	0.075	0.5	Trace	++	+++
5	"	1/16	0.075	0.5	—	—	—
6	"	1/32	0.075	0.5	—	—	—
7	Cathode C	1/1	0.075	0.5	+++	+++	+++
8	"	1/2	0.075	0.5	+++	+++	+++
9	"	1/4	0.075	0.5	+++	+++	+++
10	"	1/8	0.075	0.5	+	++	+++
11	"	1/16	0.075	0.5	—	Trace	++
12	"	1/32	0.075	0.5	—	—	—

NOTE.—The fluid of the center chamber was not appreciably different in hemolytic power from that of the cathode chamber.

TABLE VIII.

Hemagglutinin.—Cell, Fig. II, with rabbit v. horse serum (56°), undiluted. No complement added.

Current four hours. Amperes, 0.27.

	Agar Extract 0.5 c.c.	Horse Cells	Agglutination in		Agar Extract 0.5 c.c.	Horse Cells.	Agglutination in	
			30 Min.	3 Hours.			30 Min.	3 Hours.
1	Anode extract 1	0.5	—	—	Cathode extract 1	0.5	++	+++
2	" " 2	0.5	—	—	" " 2	0.5	+	+++
3	" " 3	0.5	—	—	" " 3	0.5	—	+
4	" " 4	0.5	—	—	" " 4	0.5	—	—

RESULTS OBTAINED BY THE INJECTION OF PLACENTA INTO ANIMALS OF THE SAME AND OF DIFFERENT SPECIES.

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Our anatomical knowledge of the various stages of pregnancy, except in its very earliest period, appears to be nearly complete. The clinical data dealing with the physiology and pathology of pregnancy and the puerperium are also numerous and well understood; but when we seek to obtain information about the agencies which govern the organism during the many changes incident to gestation, we meet with nothing but conjecture and hypotheses. The numerous, but often disconnected facts, upon which these various hypotheses have been founded, are based upon observations obtained at the bed side, the autopsy table, from chemical researches and animal experimentation. In view of these widely differing sources, it is not surprising that contradictions and gaps are numerous.

The object of this study has been to investigate experimentally whether actual proof can be found, that the chorionepithelium (the placenta) exerts a specific influence, whether trophic or other, upon the maternal organism. Two ways of demonstrating such an influence are at our disposal. The one consists of the injection of placental tissue into females of the same species, followed by examination of their generative organs, in order to ascertain whether any anatomical changes, particularly such as are found during gestation, have been produced. The other method is the injection of placental tissue into animals of the same and of other species with the object of producing a specific biological reaction, which can be demonstrated by various methods. In order to acquaint the reader with the current ideas entertained on this subject it will be necessary to refer to some of the literature.

It is generally conceded that the ovary instigates and regulates the periodical menstrual cycle, which is physiologically interrupted only during pregnancy and lactation until the woman reaches her menopause. According to the Born-Fränkel theory (1) which, however, from recent investigations must be accepted with some reserve, the corpus luteum exerts a powerful influence upon the nidation of the ovum. Halban (2) thinks it likely that the ovary and the corpus luteum elaborate products which are antagonistic to one another. When in the right proportion (this would be during gestation), each directly neutralizes the actions of the other. During this time, therefore, some other influence would have to bring about the numerous changes observed. Halban ascribes to the placenta this temporary governing or trophic function. Many observations have definitely shown that nervous influences play no rôle (section of the cord, lactation in transplanted breast tissue, etc.,).[1] Halban has accumulated numerous clinical observations upon which he has built up an ingenious working theory. The placenta, according to this author, causes the hypertrophy and hyperemia of the uterus and of the neighboring organs. In response to the same stimulus the decidual reaction occurs in the uterine mucosa and sometimes in other tissues, and likewise occur the hyperemia and hypertrophy of the breasts. When the placental influence is removed, as after labor or abortion, the pelvic organs undergo a marked involution, whereas on the other hand the previously hypertrophied but inactive breasts begin to secrete. The socalled "menstruation" of the newborn, and the "milk" in the breasts of infants are also ascribed, by the same author, to the withdrawal of the placental influence. Many facts and interesting observations are adduced in this paper, but on the whole Halban's theory has not met with general acceptance. The sole experimental confirmation is that of Miss Lane-Claypon and Starling (3) who

[1] Eckhard, "Beiträge zur Anatomie und Physiologie," *Eckhard's Beitrage*, 1858, i, 1, sectioned the nerves leading to the breast in goats, and found that the secretion of the milk went on unchanged.

Ribbert, *Archiv für Entwicklungsmechanik*, 1898, vii, 688, transplanted the breasts of a young guinea-pig into subcutaneous pockets in the ears. During a subsequent pregnancy the transplanted organs were found to contain functionating acini.

found that the extract derived from a rabbit's placenta, or fetus, repeatedly injected into virgin rabbits produced an hypertrophy of the breasts.[2] The incomplete experiments I have performed along somewhat similar lines will be referred to later.

Another group of investigators have sought for the true explanation by means of anatomical investigation. Chief among them is Schmorl (4) who has with great regularity found trophoblastic elements in the maternal organs. Rarely in normal women, almost regularly in eclamptics, he has discovered emboli, composed of syncytium, in the blood vessels of the lung. Kollman (5) has shown that syncytium undergoes dissolution when subjected to the maternal tissue fluids.

A third group of investigations are based purely upon experimental work, seeking to apply the Ehrlich side-chain theory to solve the problem. The activity in this line of research is so great that new observations are constantly cropping up, and consequently older conceptions will require frequent revision or rejection. Some of the older work has been made doubtful by more recently acquired knowledge. Much of this work unfortunately is still quoted and used in support of theories and far reaching conclusions.

Basing their ideas on the observations of Schmorl and Kollmann, Scholten and Veit (6) set out to prove experimentally, that fetal syncytium is dissolved by the maternal organism. It had been known that the organism reacts to the injection of foreign cells by elaborating a cytolysin which brings about the disintegration of the invader. Animals (rabbits) were injected with human placenta; their serum dissolved placental cells in vitro. Liepmann, who will be referred to immediately, was unable to obtain similar results.

Kawasoye (7) found that human placental cells when placed in the serum of gravid women produced a slight cloudy precipitate and underwent a partial solution. His controls which were kept in sodium chloride solution were, however, quite inadequate.[3]

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[2] Lane-Claypon and Starling believe that the placenta and the fetus elaborate an *hormone* which produces the breast changes. They passed the fetal or placental maceration through a Berckfeld filter and injected the fluid.

[3] Kawasoye's experiments may be divided into three groups:

1. A placental antiserum was obtained by injecting human placenta into rabbits. Large placental injections caused death, small ones albuminuria; a

When it first became known that the injection of foreign (artfremdes) proteid produced a reaction in the living organism and could be demonstrated by a visible precipitate, many investigations along these lines were undertaken. If proteid (serum, organs, red blood cells) of a certain species are repeatedly injected into an animal of another species, the serum of the immunized animal becomes cloudy when the serum or the organ extract of the animal used to produce the immunization is added in the proper proportion. The value of this reaction is generally accepted and it has great forensic importance [Uhlenhuth (8)]. It was not only thought, at first, that the reaction applied to the proteid of a given species, but it was believed that a given organ produced an even more specific serum. This refinement has, however, not stood the test of further research, and reaction only for species can in most instances be looked for [Rostoski (9), Sata (10)]. Liepmann (11) announced that rabbits injected with human placenta produced a serum, which contained precipitins for placenta or placental blood. If either of these tissues were added to the placental immune serum, a precipitate was formed. In a later communication (11) he acknowledged that the washed placenta used for immunization necessarily contained general body proteids (connective tissue, red blood cells) as well as the special placental cells, and that, to exclude a general "human reaction" a previous partial precipitation with the serum of a non-gravid subject and clearing by centrifugalizing was required, to exclude this source of error. Several other investigators [Weichardt (12), Opitz (13), Wormser (14)] have obtained negative results in repeating Liepmann's work; while Kawasoye and R. Freund (15) agree with his findings.

Veit (16) in an interesting monograph has based an elaborate theory which seeks to account for all the phenomena of pregnancy,

tolerance could be obtained. Precipitine reaction was positive with the blood of gravida, with retroplacental and cord blood, and with placental extract but negative with blood from the non-gravid and males.

2. The blood of gravid women dissolved placenta in vitro.

3. Albuminous urine of gravid women also produced an antiserum when injected into rabbits. The action of this serum was weaker and less specific than that of placental antiserum.

both those in the mother as well as those of the fetus, largely upon the above quoted works of Scholten, Kawasoye, Liepmann, Kollmann, and Schmorl. The main conclusions, germane to this branch of the subject, are of such general interest that they cannot be omitted.

In broad outline Veit's explanation is as follows: Schmorl has demonstrated syncytium in the lungs of the mother. Liepmann has furnished the biological proof that syncytial constituents are present in the blood of pregnant women, and this fact substantiates Veit's assumption that the placental proteid follows the laws of antitoxin formation and conforms to the side chain theory. Kollman has shown anatomically that syncytium is dissolved by the maternal blood; this solution presupposes a solvent chemical substance in the blood—a lysin. Veit bases all that is to follow upon the assumption "*that there is a continued entrance of syncytium from the intervillous space into the veins of the mother, and a subsequent lysis of the syncytium*" from the very beginning of pregnancy to its termination. Veit's own investigations (17) have convinced him that purely physico-chemical processes (differences in osmotic pressure) do not account for the absorption of salts and proteids by the fetus. Absorption is attended to by the villi, which dip into the maternal blood and show a selective affinity for certain substances only. This selective activity has been demonstrated by Ascoli (18) who proved that egg albumen though present in the blood of the mother did not appear in the circulation of the fetus, and by Schenk (19) who noted quantitative differences in the hemolysins and agglutinins of mother and child.

A possible explanation, according to Veit, is that the nucleus of the syncytium may control the function of assimilation, and the cell protoplasm that of excretion. When the syncytial cell is filled with excretory substances it is cast off from the villus and falls a prey to solution in the maternal blood.

The absorption of iron, which is not in solution, but is an integral part of the hemoglobin molecule and is enclosed by the red blood cell, is brought about by a similar reaction. The lysin acts as an intermediary between the red blood cell and the syncytium, and when this action occurs in the intervillous space the erythro-

cyte is bound to the functionating syncytium of the villus. Thus hemoglobin is removed without producing general hemolysis. Hemoglobin has actually been demonstrated in the villi by Hofbauer (20).

Referring to the pathological phenomena of pregnancy Veit offers the following explanation: The albumin found in the urine of patients suffering from albuminuria of pregnancy, is a true heterologous albumen, such as Liepmann pronounced it to be; it is due to dissolved syncytium which has entered the maternal blood stream in large amounts. If the quantity is very great, the syncytium which is then only partly neutralized acts as a toxin on the kidneys, and produces an actual nephritis. The proof of the nature of this albumin lies in the fact that the specific precipitine reaction is obtained with such urines (Kawasoye). When the syncytium is in greatly excessive amount it no longer acts as a merely local poison, a renal irritant, but produces systemic symptoms which manifest themselves in the eclamptic seizure. Possibly other morbid conditions of pregnancy, such as hyperemesis, icterus, morbus maculatus, etc., are due to this same cause.

The views of Veit, as set forth in the article which has been mentioned, cover every branch of the subject, and in building up his hypothesis he has made use of practically all the work, which has been done along these lines. In the first place he has accepted the anatomical findings of Schmorl and Kollmann.[4] The discoveries of Schmorl may be considered conclusive.

The biological experiments, which are believed to show that the syncytium not only can, but does enter the maternal circulation, acts as a toxine on the tissues of the mother, and stimulates receptor formation, have not been convincing. In opposition to the positive findings of Veit and Scholten, Liepmann, and Kawasoye, Weichardt, Opitz and Wormser obtained negative results, as already stated. Veit has apparently not attached sufficient weight

[4] The work of Kollmann, as far as it bears upon this point, is not convincing. The material was obtained from a fetus three weeks old; some specimens were from pregnant monkeys. He interprets the vacuolization and disintegration noted in the syncytium, as due to lytic processes. It may be said, that changes, such as he pictures, are to be found in almost any rapidly multiplying tissue, and certainly do not necessarily imply any specific solvent process.

to the negative findings, particularly if we take into consideration the fact that some of the positive results were not constant or strictly specific.

Without entering into the merits of Veit's interesting hypothesis, which may yet prove to contain the true explanation of the phenomena of pregnancy, I have sought to repeat some of the work performed, and have used in part the methods already employed by others, in part newer or different methods.

1. To investigate Halban's placental theory three rabbits were repeatedly injected with fresh placenta of pregnant rabbits removed by operation just before term. These animals were used: (*a*) anatomically to study the uterus, ovary and tubes; (*b*) biologically to test the serum for precipitines. The difficulty in obtaining sufficient material for injection made it necessary to cut short this series of experiments somewhat summarily.

2. To control the work performed by Liepmann, etc., three groups of rabbits, each group composed of two animals, a male and a female, were injected repeatedly with the following materials: (*a*) A solution of nucleo-proteid obtained from the human placenta. This was tested for precipitines, and for deflection of complement by the method of Neisser and Sachs. (*b*) A maceration of human placenta made blood-free by washing in plain running water; it was tested by the same methods. (*c*) A maceration of human placenta made as bloodless as possible by washing with large quantities of normal saline solution; it was tested for precipitines, deflection, cytolysis, etc.

RABBITS INJECTED WITH PLACENTA OF RABBITS.[5]

A double purpose was kept in view in this series of experiments. Firstly, if placental tissue should prove to have an inherent specific action such as Halban ascribes to it, repeated intraperitoneal injections ought to produce distinct changes in the genital system. Such changes in the mammary glands were reported by Lane-Claypon and Starling a number of months after my experiments were

[5] This group of experiments was performed in the Pathological Laboratory of the College of Physicians and Surgeons during the months of April and May, 1906. I desire to express my obligations to Professor T. M. Prudden for the courtesies extended to me.

completed. The duration of pregnancy in the rabbit covers thirty days, therefore it would seem necessary to extend the injections over a period of about that length.

Secondly the use of the serum obtained, if potent, would avoid a disturbing factor not to be overcome if the injections were made into an animal of another species, namely hemolysins, and "a general species reaction," for the tissues of the animal used to produce immunization. The work of Kraus and Ludwig (21), and still more recently that of Schultz (22), shows that iso-hemolysins cannot be obtained in rabbits. It might be supposed that experimentally, at least, iso-syncytiolysins, would also not be formed. However the analogy is not quite exact, for in virgin animals (such as were used in this series) the placenta, if it differ biologically from other tissues of the body is a foreign substance. An article by Ed. Martin (23) has just appeared reporting attempts to prove the presence of iso-hemolysins and iso-agglutinins obtained in the following way. Twelve rabbits were operated upon, one half of the pregnant uterus (which in the rabbit is didelphous) being removed, and 0.5 grams of the mashed placenta at once injected into the ear vein of the same animal. In none of the experiments were either hemolysins or agglutinins obtained.

Technic.—Three young female rabbits were employed. A number of other rabbits were allowed to be impregnated at regular intervals, and the gravid uterus taken out at about the twenty-second to twenty-fifth day of gestation. The six to eight placentæ obtained at each operation were at once cut up and mashed in a mortar, the maceration thinned out with an equal volume of normal salt solution and ten cubic centimeters injected intraperitoneally through a large needle into the experimental animals. Throughout, aseptic precautions were employed and no infection occurred. After each injection the animals did not take food for a few hours, but showed no other ill effects; however, during the entire course of the experiments they lost some weight. Nine injections were given to each rabbit at three-day intervals. On the fifth day after the last injection the animals were bled to death and the clear serum obtained in the usual way. The pelvic organs were at once placed in five per cent. formalin and prepared for examination by embedding in celloidin.

The material for histological examination consisted of blocks taken from at least four levels of the tubes and uterus, and longitudinal sections of each ovary. The controls consisted of material taken from normal animals killed in the laboratory during the time in which these experiments were performed. The result of

the examination of these organs can be summed up by stating that there was not the slightest difference between the organs of the injected and of the control animals. No decidual reaction, not even an increased vascularity, was found. Macroscopically no difference in size or appearance could be noted.

The serum tests were performed as follows:

Fresh serum from the injected rabbits was taken in increasing dilution (see Table), placed in small test tubes, and to this serum was added extract of rabbit's placenta (allowed to stand forty-eight hours and prepared in the cold) of various strengths. In another series a loop full of maceration of washed rabbit's placenta was added to the immune serum. Both series were at once brought to a uniform volume of two cubic centimeters by adding normal salt solution. They were placed in the thermostat for one hour and then in the ice-chest for from twelve to twenty-four hours. In none of these series did a precipitate appear.

Anti-serum.	Placental Extract or Placental Tissue		
0.2 c.c.	0.1 c.c.	0.05 c.c.	0.01 c.c.
0.1	"	"	"
0.05	"	"	"
0.2	—	—	—
—	"	"	"

RABBITS INJECTED WITH HUMAN PLACENTA.[6]

Placental Nucleo-Proteid.—The work of Bierry and Meyer (24) and that of Beebe (25) and others lead one to expect that the nucleo-proteid, of a tissue, yields a more specific immune serum and gives less "general species reaction" than the serum obtained from injecting the washed organ. Levene (26) however says, that extracts made from various constituents of red blood cells and used for immunization, either give no hemolytic sera or sera far less potent than those obtained from the entire red blood cells. Pearce and Jackson (27) in a paper published recently, claim that a repetition of Beebe's work has given negative results. These investigators have made a very complete series of experiments from

[6] Almost all the human material used for these and the succeeding experiments was obtained through the kindness of Dr. George Ryder, resident obstetrician of the Sloane Maternity Hospital.

which they conclude that the nucleo-proteids act as mild toxic agents, affecting chiefly the excretory organs, and that they do not produce specific anti-sera. They say that Ehrlich and Morgenroth have shown that anti-sera are produced not by specific cells, but by specific free receptors. Specificity in the morphological sense cannot be demonstrated. The sole criticism that can be applied to the work of Pearce and Jackson is that they have prepared the nucleo-proteid by the " hot " method, which necessitates bringing the mashed organ to the boiling point! Such a method is inadvisable if biological reactions are to be employed. These authors did not attempt to make precipitine tests, but studied the effect of the injections upon the organs in vivo and also histologically.

Technic.—Many (ten to twelve) placentæ were passed through a meat machine and washed in large quantities of 0.9 per cent. saline solution. The blood-free tissue, to which a 0.5 per cent. sodium carbonate solution in the proportion of 1 : 3 had been added, was placed in the ice chest for twelve hours. Microscopical examination of the last wash water still showed the presence of red blood cells in small number, about one hundred being present in the ordinary low power field. The extract was next filtered through several thicknesses of gauze and the nucleo-proteid precipitated by means of a slight excess of acetic acid. The precipitate was now washed by decantation and attempts [illegible] redissolve it with sodium carbonate solution. It was found that unless [illegible]ocess was hastened, the carbonate solution did not act as a solvent, but a decinormal sodium hydrate caused solution. Reprecipitation and redissolving completed the process.

No exact analysis of the amount by weight represented by each unit of solution was determined, but as from twenty-five to thirty cubic centimeters of very concentrated solution were given, at each injection, a considerable quantity of the nucleo-proteid was used.[1] Dr. P. Levene of the Rockefeller Institute had the kindness to make a Phosphorus determination on the nucleo-proteid used in these experiments. The phosphorus percentage proved to be 0.35.

The solution was injected into two rabbits, a male and a female, intraperitoneally at intervals of about five days, seven injections in all being given to each animal. Serum was taken after the fifth injection and again after two additional injections. The animals flourished during the course of the experiments and gained in weight.

The serum of these rabbits was tested, while fresh, with placental extract (human placenta) and with bits of washed placenta, just as in the previous series. No precipitin reaction was obtained, nor did human blood-serum produce any result.

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[1] The first set of sera obtained from these animals were inactivated at 55° C. Noguchi's paper (*Journal of Experimental Medicine*, 1906, viii, 726) refers to the substances which he calls "protectines," or antilysines, formed when sera are heated above 56° C. To avoid all possibility of error from this source the subsequently obtained sera were all inactivated at 52° C.

Another portion of each serum was inactivated at 55° C., for fifteen minutes and then tested by the method of Neisser and Sachs (28) for deflection of complement. This method is regarded as even more delicate than the precipitin reaction, although it is more troublesome and is somewhat capricious.

The rationale of the reaction is as follows: An indicator consisting of an independent hemolytic system is used. In my experiments rabbit's serum made lytic to hen's corpuscles by repeated injections of the washed corpuscles of hen's blood was employed. This anti-hen's serum was inactivated by heat. The *other* component of this series was hen's corpuscles diluted to a strength of five per cent. If fresh guinea-pig's serum (complement) is added, hemolysis is complete within one hour at a temperature of 37° C.

The second or main components of the reaction are the following: The placenta anti-serum (inactivated) is supposed to contain the amboceptor or specific binding-body if such is elaborated. The organ extract used for immunization must contain corresponding receptors; it is called the antigen. If to these two bodies combined in the proper proportion (see Table) a sufficient but not too great quantity of complement is added, the amboceptor should serve to bind the complement to the receptors. As the amboceptor is specific, it will serve only to bind complement to corresponding receptors. In other words, *should* an appropriate antiserum be obtained, the free complement is bound to the receptors, and when the hemolytic series (which contains no complement) is added, no hemolysis results. As actually performed, the amboceptor, antigen and complemer [illegible] added in varying proportion, placed in the thermostat for one hour to [illegible] deflection of complement to take place, and then the second series (hen's antiserum and hen's corpuscles) are added. The complete mixture is then again placed in the thermostat for two hours more. The result, *i. e.*, hemolysis complete, incomplete or absent, is read. The tubes are put in the ice chest for from twelve to twenty-four hours and the final reading taken.

In my experiments various quantities of the components were tried. The following table shows the proportion which was most economical of anti-serum and yet gave the greatest number of proportions of amboceptor and antigen. Throughout, just sufficient complement was used to assure complete hemolysis. The necessary quantity of complement was determined anew each time fresh complement was used.

To these and to all succeeding reactions enough normal salt solution was added to bring the total volume to four cubic centimeters.

When negative results were obtained in reactions similar to those described in the above tables, other experiments were performed, using as much as 1.0 c.c. of anti-serum, and 0.5 c.c. of placental extract. Only after repeated trials of these various proportions, was a negative result accepted.

Anti-serum (Amboceptor).	Placental Extract (Antigen).	G. P. Serum (Complement).	Hen's Anti-serum.	Hen's Corpuscles 5%.
0.2 c.c.	0.01 c.c.	0.04–0.025 c.c.	0.01 c.c.	1.0 c.c.
0.1	"	"	"	"
0.05	"	"	"	"
0.01	"	"	"	"
0.005	"	"	"	"
0.001	"	"	"	"
0.0005	"	"	"	"
0.2	—	"	"	"
0.2	—	"	—	"
—	0.01	"	0.01	"
—	—	"	"	"
—	—	"	—	"
—	—	—	0.01	"
—	—	—	"	"

Placental Extract.	Anti-serum.	G. P. Serum.	Hen's Anti-serum.	Hen's Corpuscles.
0.1 c.c.	0.1 c.c.	0.04–0.025 c.c.	0.01 c.c.	1.0 c.c.
0.05	"	"	"	"
0.01	"	"	"	"
0.005	"	"	"	"
0.001	"	"	"	"
0.0005	"	"	"	"
—	"	"	"	"
—	"	"	—	"
0.1	"	"	0.01	"
0.1	—	—	0.01	"
—	—	0.04–0.025	"	"
—	—	—	"	"
—	—	—	"	"

Before proceeding to the complete reactions, which the tables show, it was necessary to determine in each case that the given anti-serum was inactivated and was neither in itself hemolytic or anti-hemolytic. Two of the anti-sera (those obtained from human placenta washed with normal salt solution) proved to contain a small trace of *amboceptor for hen's corpuscles.* This disturbing factor was removed by treating these sera, for one hour at 37° C., or over night at 0° C., with a great excess of hen's corpuscles and using the supernatant clear fluid after centrifuging. Variously prepared extracts were tried. One set was obtained by placing the macerated washed placenta, diluted with normal salt solution 1:3 for forty-eight hours in a shaker, the other in the thermostat for forty-eight hours. A second set of extracts were made using plain distilled water. As no great differences were found, the extract finally used was the one prepared with salt solution in the thermostat. Toluol was added to prevent bacterial growth, and

this antiseptic was later removed by evaporation. The extract was always tested for its hemolytic action and a quantity far below this point used in the reactions.

The four nucleo-proteid sera obtained from the two injected animals showed no deflection of complement. In other terms, the presence of a specific antibody could not be demonstrated either by the precipitin or by the reaction for deflection of complement.

Placenta Washed in Running Water.—As not all the red blood cells could be removed by washing the placenta on a filter with normal salt solution, and it was not feasible to obtain the placentae so early that they could be exsanguinated by washing through the umbilical cord, the finely chopped placenta was placed in a large gauze bag and attached to the cold water faucet. By these means the tissue became practically blood free in a short time, the last wash water showing very few red blood cells, or rather "shadows." The drawbacks attached to this method were fully realized, but it was hoped to control the results by experiments which follow.[8] The placental tissue was then ground in a mortar with sand, and a thick, yet finely divided placental suspension prepared with sterile salt solution. Cultures of each batch of suspensions either showed no growth, or in the case in which growth occurred, an apparently non-pathogenic saprophyte was found.

Three rabbits, two males and one female, were used. One male died after the second injection, autopsy showing a peritonitis due to accidental perforation of the intestine. The other two animals received, respectively, six and eight injections intraperitoneally. The one which received eight injections was given 15 c.c. each time; the other was given from 25 to 30 c.c. The first animal gained over 200 grams in weight, the second lost nearly 400 grams.

The two sera obtained were tested both by the precipitin reaction and by the method of deflection of complement. *The results were absolutely negative.*

Placenta Washed in Normal Salt Solution.—The previous ex-

[8] Washing the tissue with a solution which was not isotonic made it not unlikely that at least some of the antigen would be carried away. Yet by this method a more complete removal of the red blood cells was obtained, and the microscopical examination of the tissue showed that enormous quantities of well preserved placental cells remained for injection.

periments, in which the placenta was rendered practically blood free, at the cost of losing at least some of its active constituents, resulted negatively. As a check and control, the placentae in the series now to be detailed, were first chopped fine with a meat machine, and then each placenta was washed on a filter with ten liters of salt solution, with constant stirring. A careful lookout was kept for all visible blood clots and these were removed. The last wash water appeared clear macroscopically, but under the microscope, from twenty-five to one hundred red blood cells to the low power field were noted. I have been unable to find any reference, in the literature, to the minimum quantity of blood necessary to produce an immune serum. The further treatment of the material in no way differed from that employed in the previous series.

Technic.—Three rabbits were used at the outset, two males and one female; but one male was so seriously injured in a fight with the others that the injections had to be discontinued.

Both the remaining rabbits received nine injections each, at intervals of about five days, from twenty-five to thirty cubic centimeters being introduced intraperitoneally. The animals were bled after the seventh and again after the ninth injection. Both gained markedly in weight during the treatment. In spite of the injections, the *female conceived and bore four normal young* during the course of the experiment. When bled to death she was found to be again pregnant (about twelve days gravid).

Of the four sera, the one serum obtained from the female (after the seventh injection) was rendered useless by being overheated, due to a faulty thermometer. The three other sera were tested for:

1. Precipitin reactions.
   - *a.* Precipitin reactions with placental extract.
   - *b.* Precipitin reactions with cord blood serum.
   - *c.* Precipitin reactions with retro-placental blood serum.
   - *d.* Precipitin reactions with normal blood serum from a male.
   - *e.* Precipitin reactions with placental extract after saturation with male blood serum and with human red corpuscles.
   - *f.* Precipitin reactions with urine (non-albuminous) of gravidæ.
   - *g.* Precipitin reactions with urine (albuminous) of gravidæ.
   - *h.* Precipitin reactions with urine of a male, containing albumin.
2. Reactions for deflection of complement.
   - *a.* Reaction for deflection of complement with placental extract.
   - *b.* Reaction for deflection of complement with normal human blood serum (male).
3. Agglutination reaction with human corpuscles.
4. Hemolysis reaction with human corpuscles.
5. Cytolytic reactions.

a. Cytolytic reactions with emulsions of placental cells.
b. Cytolytic reactions with small pieces of placental tissue.

The results obtained by the various tests follow, and as the three sera have given the same results, in approximately the same dilution, only one set of records will be described.

1. PRECIPITIN TESTS.

a. A faint but positive reaction was obtained with washed placental extract (0.05 c. c.) and the immune sera in dilutions of from 1 : 10 to 1 : 20 corresponding to 0.2 to 0.1 c.c. *Normal rabbit serum gave no precipitate to placental extract or to the other substances tested in Experiments e to h.*

b. The cord blood, from two cases, gave similarly positive results.

c. Retro-placental blood serum, from two cases, gave a distinct but weaker reaction.

d. Normal human blood serum (from a male) gave a precipitate, in the same dilutions as in Experiment *a.*

e. Partial precipitates were sought for. To the immune serum was added an excess of normal human serum. After the precipitate had formed, the fluid was cleared by centrifuging, and then placental extract added as in Experiment *a.* No precipitate appeared in the tubes to which placental extract had been added, nor in those into which more human serum was placed, although even stronger solutions than in Experiment *a* were used. After it was observed that the antisera were hemolytic, another method for obtaining a partial reaction was tried. An excess of a five per cent. solution of human corpuscles was added to the antisera and kept in the ice chest for twenty-four hours. To the clear solution was added placental extract and human serum, in the same proportions as in Experiment *a.* No precipitate was obtained. The control with more human blood cells showed no further hemolysis.

f. The urines of six gravid women (containing no albumin) were tested, using 0.1 to 0.05 c.c. of urine, to the same quantity of antiserum as in the other experiments. No precipitate resulted.

g. The urines of two gravid women (containing much albumin, one in the pre-eclamptic stage, the other toxemic) were tested. The one containing the most albumin gave a negative, the other a faint but positive reaction.

h. The urine of a man containing much albumin gave a faint but positive reaction.

The precipitin reaction in all these experiments showed a faint but unmistakable *human reaction, but no specific placental reaction.*

2. TESTS FOR DEFLECTION OF COMPLEMENT.

For experimental details see heading "Nucleo-Proteid Tests."

a. For all three sera it was found that with from 0.004 to 0.001 c.c. of antiserum, and 0.01 c.c. of placental extract, using slightly more than the minimum amount of complement required (0.03 to 0.025 c.c.), hemolysis was incomplete or absent.

b. With human serum (male) a positive reaction was obtained in much greater dilutions (down to 0.001 c.c. of human serum with 0.003 to 0.001 c.c. of antiserum).

Deflection of complement was more strongly marked in the case of normal blood serum than in that of placental extract.

3. Agglutination Tests. Agglutination of a five per cent. solution of normal human blood corpuscles was obtained in dilutions of the antisera between 1 : 6 and 1 : 12, 0.5 c.c. of corpuscles being used. Normal rabbits' serum failed to agglutinate.

4. Hemolysis Tests. Hemolysis of 0.5 c.c. of a five per cent. solution of human corpuscles was obtained in dilutions of from 1 : 5 to 1 : 12 of the antisera, the controls with normal rabbits' serum again proving negative.

5. Cytolysis Tests.

a. Suspensions of placental cells, obtained by the method used by Flexner and Noguchi (29) in their cytolytic experiments, were added to 0.5 c.c. of the undiluted antisera, to normal rabbits' serum and to normal sodium chloride solution, kept in the incubator at body temperature for one hour, and then in the ice chest for twelve hours. Unstained and stained specimens of the cells were examined. No very marked differences were noted. On the whole the specimens kept in normal rabbit serum showed the best preservation. In all the others the chromatin network was found more coarsely granular and the nuclear outline less distinct; while in the unincubated specimens, made for control, immediately after preparing the cell emulsion, there were numerous Langhans' cells in addition to the syncytium; in the incubated specimens the syncytial cell complexes greatly preponderated.

b. A similar set of experiments to the foregoing were performed, using small pieces of placental tissue instead of isolated cells. All the specimens were transferred to a five per cent. solution of formalin embedded by the celloidin method, cut, and stained with hematoxylon and eosin. No differences were noted except that as in the previous series the specimens preserved in sodium chloride solution showed less perfect staining qualities. Otherwise the placental tissue was perfectly normal.

CONCLUSIONS.

The injection of rabbits' placenta into rabbits produces no isoprecipitins.

From the incomplete experiments performed it would appear that placental injections into animals of the same species cause no changes in the generative organs. Further research into this question will be pursued.

The injection of human placental nucleo-proteid, prepared from placental tissue made nearly blood free, does not produce an antiserum. This result confirms the conclusions of Pearce and Jack-

son, that nucleo-proteids act merely as mild toxic agents, without specific qualities.

The injection, into rabbits, of human placental tissue, rendered practically blood-free, fails to produce any specific reaction. This confirms the view that the serum reaction following the injection of cells into a foreign organism is largely due to the blood contained in the injected tissues.

The injection into rabbits, of the human placenta, made nearly blood-free, produces a weak "human reaction" which can be demonstrated by the reactions for precipitin, deflection of complement, agglutinin, and hemolysis. *No specific placental reaction can be shown.* This is in strict accord with the view that cytotoxines are not specific; that there is no morphological specificity.

The anti-sera obtained showed no cytolytic action; therefore no specific syncytiolytic action could be demonstrated.

If the information obtained in this investigation is applied to the theory of Halban, it will be noted that no experimental proof of the specific action of placental tissue upon the female generative organs could be demonstrated. The number of experiments performed, bearing upon this one point, were however too few to permit of a definite and final opinion.

The work dealing with Veit's ingenious hypothesis was more complete and carried out by many methods, which would necessarily act as a check upon one another. As the results of all these experiments were in complete harmony, I feel justified in making a positive statement that *no experimental proof of a specific placental immune reaction can be demonstrated by our present biological methods.* Whether Veit's hypothesis, thus deprived of its biological proof, must in consequence be discarded, is a question which I do not consider myself competent to answer.

In conclusion I desire to thank Dr. Simon Flexner, Director of the Rockefeller Institute for Medical Research, for extending to me the privileges of his laboratories. I also wish to acknowledge my indebtedness to Drs. Jobling, Noguchi and Levene for the frequent advice and assistance I have received from them.

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THE NATURE OF THE ANTITETANIC ACTION OF EOSIN.

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In a previous publication[1] Dr. Flexner and I gave the results which we obtained from a study of experimental tetanus in rats and guinea pigs subjected to the influence of certain photodynamic anilines. To recall briefly the main facts brought out there, it may be stated that a solution of eosin, of a certain concentration, destroys in vitro the haemolytic and tetanospastic principles of tetanus toxin, and when applied to the site of inoculation of tetanus spores on threads, or tetanus-toxin, either entirely prevents the appearance of the tetanic symptoms, or delays the appearance and diminishes the effect, and, in rats, sometimes prevents the fatal issue. In a great number of instances in which the eosin is applied directly to the spore-infected area in rats, the lives of the animals can be saved. The next step in this investigation was the determination of the mode of action of the eosin through which the effects already described are produced.

The present paper deals with the manner of action of eosin upon *Bacillus tetani* without and within the body of animals. The subject falls naturally, therefore, into the two main divisions of the action of eosin upon the tetanus bacillus *in vitro* and *in vivo*.

THE ACTION OF EOSIN UPON THE TETANUS BACILLUS IN VITRO.

Relating to this topic, the following points were considered: (1) the influence of eosin upon the germination, growth, sporulation, morphology, and toxin production of *B. tetani*; (2) the bactericidal and sporicidal effects of eosin upon *B. tetani*; (3) the viability of *B. tetani* in eosinized media; (4) the toxin-producing power of *B. tetani* exposed to the action of eosin.

[1] Flexner and Noguchi, *Jour. of Exper. Med.*, 1906, viii, 1.

Cultivation of Bacillus tetani in Eosinized Culture Media. (*a*) *Eosinized Glucose Bouillon.*—Two kinds of eosin—"rein" and "gelb"—were employed in strength of 0.001 per cent., 0.01 per cent., 0.1 per cent., and 1 per cent. A vigorous culture of the tetanus bacillus was inoculated and incubated at 37° C. in an atmosphere of hydrogen for 10 days.

The control cultures were abundant; they contained typical bacilli forming few threads and showing few spores. The growth in 0.001 per cent. eosin was equal to the control, but microscopically the thread forms predominated and no spores whatever could be found. The growth in 0.01 per cent. eosin was equal to the control, but upon microscopic examination only asporogenous thread forms were seen. The growth in 0.1 per cent. eosin was slight and showed single, asporogenous bacilli. No growth whatever took place in the 1 per cent. eosin medium.

The first effect which the eosin produces, apparently, is to suppress sporulation of the bacilli, and the next is to increase thread formation. The limit of concentration of eosin which permits restricted germination is between 0.1 and 1 per cent., and is probably not far from the former concentration. A later determination placed this limit at 0.2 per cent. The cultures prepared as described were filtered through porcelain and tested for toxicity upon rats. The number of m. l. d. per cubic centimeter of filtrate was as follows:

Control	= 1000 m.l.d.
0.001 per cent. eosin	= 700 m.l.d.
0.01 per cent. eosin	= 100 m.l.d.
0.1 per cent. eosin	= 1 m.l.d.
1.0 per cent. eosin	= 0 m.l.d.

No remarkable difference in the properties of the two kinds of dye was noticed. The reduction in toxicity is greater than the reduction in growth caused by the eosin. The diminished toxicity has, doubtless, a two-fold origin: it arises from the diminished multiplication of the bacillus, and from the injury exerted by the eosin upon the toxin originally produced. The degree of this destruction can be deduced from the relative strengths of the filtrates from the control and the 0.001 per cent. eosin cultures, and it amounts to about 30 per cent. As the eosin strength grows, the effects upon the multiplication and toxicity increase, but not in equal ratio. A corresponding comparison of the hæmolysin (tetan-

olysin) present in the several filtrates was also made. Each cubic centimeter contained the following units:

Control filtrate contained			*C.H.D.	20	*M.H.D.	333
0.001 per cent.	" "rein"	"	"	12	"	242
0.001 "	" "gelb"	"	"	10	"	200
0.01 "	" "rein"	"	"	4	"	66
0.01 "	" "gelb"	"	"	3	"	50
0.1 "	" "rein"	"	"	0	"	0
0.1 "	" "gelb"	"	"	0	"	0

* C.H.D. = complete hæmolytic dose; M.H.D. = minimal (trace) hæmolytic dose.

Eosin destroys or suppresses the tetanolysin in the cultures as it does the tetanospasmin, a fact which could be predicted on the basis of the observations of Flexner and Noguchi.[2] Note should be taken of the greater activity in this respect of eosin "gelb" as compared with eosin "rein."

(*b*) *Eosinized Glucose Agar.*—The advantage for the study of *B. tetani* which glucose agar has over glucose bouillon is derived from the fact that in the former medium sporulation is much more abundant, and is already easily noticeable after twenty-four hours growth. At the end of seven days spores are numerous, and at the expiration of about thirty days, no vegetative bacilli remain. Hence, to glucose agar the following strengths of both eosins were added, 0.01 per cent., 0.02 per cent., 0.05 per cent., 0.1 per cent., 0.2 per cent., 0.5 per cent., and 1 per cent. Stab inoculations were made; cultivation at 37° C.

The growths were well-marked in the tubes below 0.05 per cent. strength; above this limit the growths diminished progressively with increase in concentration. On microscopical examination, it was found that 0.1 per cent. of eosin was the limit of concentration for spore formation. Below this concentration, sporulation took place; at and above it, no sporulation occurred even after three months. The number of spores in eosinized media is below the number in ordinary media; the situation of the spores in the former media is the centre of the body. Transplantation of the atypical sporulating bacilli to simple glucose agar brings about immediate

[2] *Op. cit.*

return to typical mode of sporulation. The limit of germination is for eosin " rein " about 0.5 per cent., while for complete germination, it is about 0.2 per cent. Strengths above 0.5 per cent. inhibit vegetation of spores, but they do not kill the spores. Eosin "gelb" is somewhat more inhibitory. Concentrations above 0.02 per cent. prevent sporulation, but permit vegetation; above 0.5 per cent. no vegetation occurs.

(*c*) *Eosinized Tissue Bouillon.*[8]—The tetanus bacillus grown aerobically at 37° C. in tissue bouillon produces abundant spores in a few days. The sediment which forms in the cultures in a few days, is composed largely of spores. The effect of eosin " gelb " on this process was studied by adding strengths of 0.001 per cent., 0.003 per cent., 0.01 per cent., 0.03 per cent., 0.1 per cent., 0.3 per cent., 1 per cent., and 2 per cent. of the dye. The tissue absorbs a part of the dye; the cultures were incubated at 37° C. for twenty hours when first examined, and subsequently for twenty-nine days longer.

	After 20 Hours.	
Control.	Good growth; gas formation.	C.S.* single bacilli; no spores.
Eosin "gelb" 0.001 %	Idem.	C.S. chiefly single, few threads, no spores.
" " 0.003	Idem.	C.S. bacilli chiefly in chains, no spores.
" " 0.01	Less growth.	C.S. long threads only showing vacuoles and irregular contours.
" " 0.03	Idem.	Idem.
" " 0.1	Slight growth.	C.S. very few single bacilli, small number of spores.
" " 0.3	Doubtful growth.	C.S. few bacilli and spores.
" " 1	No growth.	C.S. very few spores.
" " 2	Idem.	Idem.
	After 30 Days.	
Control.	Fluid clear; deposit heavy.	C.S. all bacilli bear spores.
Eosin "gelb" 0.001 %	Idem.	C.S. numerous threads and spores.
" " 0.003	Idem.	C.S. nearly exclusively in threads; very few spores.
" " 0.01	Idem.	C.S. threads; no spores.
" " 0.03	Idem.	Idem.
" " 0.1	Slight deposit.	C.S. a few short chains; many single bacilli; few spores.
" " 0.3	No change.	
" " 1	No change.	
" " 2	No change.	

* C.S. indicates cover slip preparations stained in the usual way.

[8] Theobald Smith, *Jour. of the Boston Soc. of the Med. Sciences*, 1901, iii, 340. Tarozzi, *Centralbl. f. Bakt.*, etc., Orig., 1905, xxxviii, 619.

The foregoing observations are in conformity with those already recorded. In spite of the vigorous growth of the bacilli in this medium containing small quantities of eosin, the biology of the organisms is influenced directly as regards their power to segment and to form spores. With increasing concentrations of the eosin these effects become more pronounced, until in the concentration of 0.1 per cent. only part of the transplanted spores are enabled to germinate, and the vegetative bacilli produced by this imperfect germination are restrained from free multiplication. This process of imperfect vegetation comes, I believe, to play a very interesting part in developing immunity in inoculated rats treated with eosin, of which phenomenon I will have occasion to speak hereafter.

(*d*) *Bactericidal and Sporocidal Properties of Eosin upon B. tetani.*—Glucose bouillon cultures of the tetanus bacillus do not form spores within forty-eight hours, hence such cultures can be employed to test the bactericidal effect of strong solutions of eosin. It was found that such spore-free cultures when mixed with eosin to the concentration of 2 per cent., and kept for fifteen minutes in diffuse light, fail to grow upon replantation. If the eosin strength falls below 1 per cent., not all the bacilli are killed. Contact for twenty-four hours of vegetative sporeless bacilli with eosin in solutions above 0.1 per cent., causes their death. The bactericidal effect of eosin is increased by exposure to the sun: eosin in a strength of 0.02 per cent. can cause in eight hours the death of vegetating bacilli, when exposed directly to the sun's rays. If the experiments are made under anaerobic conditions the results are not essentially different.

The spores of the tetanus bacillus display far greater resistance. Solutions of eosin of 5 per cent. and 0.05 per cent. failed to bring about their destruction after exposure under aerobic and anaerobic conditions to the direct rays of the sun for thirty hours.

(*e*) *Viability of B. tetani in Eosinized Media.*—Cultures eighty-eight days old of the tetanus bacillus in glucose agar containing 0.1 per cent. eosin "gelb" showed no spores. On transplanting from these cultures into glucose agar, a feeble growth containing few spores after seven days was secured. Transplantations into esoin-free media from this slight growth gave cultures which be-

haved, in all respects, in a normal manner. From this experiment the conclusion can be drawn that concentrations of eosin which are not quickly fatal to sporeless tetanus bacilli reduce in the first generation their power of reproduction but do not suffice to kill them outright even after long periods of contact.

(*f*) *Can B. tetanus be Rendered Durably Asporogenous by Eosin?*—Eosin in the strength of 0.01 per cent. reduces spore-formation in *B. tetani* and in the strength of 0.1 per cent. prevents it entirely. A strain of *B. tetanus* was cultivated in 0.01 per cent. and 0.1 per cent. successively through many generations. At each transfer to eosinized medium a control culture was made in glucose-agar to observe the point of final disappearance of the spore-bearing faculty. In the case of eosin "rein," the bacillus withstood fairly well the successive implantations into the eosinized medium, but the faculty to produce spores in ordinary media was not lost after eight generations, covering a period of three months, in eosinized glucose agar. A reduction of the spore-formation was noted in the first generations in the plain glucose medium. In the case of eosin "gelb," the bacillus survived for three generations only in 0.1 per cent. eosin-glucose-agar, and for the eight generations in the 0.01 per cent. medium. No permanent alteration of spore-bearing capacity was effected in the latter cultures.

(*g*) *Is the Toxin Producing Power of B. tetani Affected by its Growth in Eosinized Media?*—Two cultures of *B. tetani* were kept in 0.1 per cent. eosin "gelb" glucose-agar for eighty-eight days, after which they were renewed by transplantation to glucose bouillon. After forty-eight hours growth at 37° C., these cultures and suitable control cultures were filtered through porcelain. The toxicity of the filtrates was approximately equal. The deduction from this experiment is obvious: no permanent influence upon the toxin-producing faculty of *B. tetani* is exerted by long contact of eosin in concentrations below the bactericidal limit.

THE ACTION OF EOSIN UPON THE TETANUS BACILLUS IN VIVO.

The next subject of the study taken up related to the manner in which eosin acts in preventing tetanus in rats inoculated with tetanus bacilli or their spores.[4] The effects to be explained are,

[4] Flexner and Noguchi, *op. cit.*

briefly, these: rats, beneath the skin of which tetanus spore-threads are placed, regularly develop tetanus and die. If, however, the inoculated rats are treated by injections of eosin about the spore-thread, many recover, and some even fail to develop any symptoms of tetanus. Injection of the eosin in other parts of the body may delay the appearance of tetanus and the fatal issue, but does not suffice wholly to prevent them. The effect of the eosin on the local reaction to the tetanus bacilli in the inoculated rats is to be explained.

Fate of Spores Introduced into the Body on Threads.—Eighteen rats (weighing about 90 grams each) were inoculated beneath the skin of the thigh with spore-threads of *B. tetani,* free from toxin, on June 8, 1906. The six of these left untreated (controls) developed tetanus in two to six days time (average, three days). Death usually resulted on the third day after the appearance of the tetanus. Cultures were made from the threads and the liver.[5] Tetanus bacilli were recovered from the threads in all six animals and from the liver in three animals. The remaining twelve rats were treated with eosin "rein." Doses of 0.2 to 0.4 c.c. of a 2 per cent. solution were injected successively on three days, beginning immediately after the inoculation, about the thread, and intermittently (every other day) for three more injections. The treated animals did not develop tetanus, and a part of them were alive and healthy four months after the inoculation.

The first question which I asked myself is, *Do the tetanus spores germinate and multiply under the influence of the eosin, and what becomes of the germinated bacilli?*

Experiment. Rat No. 7. A part of the spore-thread was removed forty-eight hours after inoculation and during the eosin treatment. Cover slips prepared from it showed many spores, a small number of bacilli and a few leucocytes. A second portion of the thread was removed on the ninth day (day of last eosin injection). Cover slips showed very few bacilli and many spores and leucocytes. Cultures gave pure growths of *B. tetani.* On the thirteenth day, the remainder of the thread was removed. Now only spores could be found, and leucocytes were no longer present. The rat remained well.

Rat No. 8. Same treatment; examination on fifth day; among many spores a small number of bacilli—vegetative forms—were seen.

[5] Tarozzi, *Centralbl. f. Bakt.*, etc., 1906, xl, 305, 451.

The study of the spore threads in the two inoculated rats show conclusively that the injections of eosin do not wholly prevent the germination of the spores, but that the germination is largely suppressed; and it also renders the view very probable that of the germinated spores few or none multiply in the eosinized tissues, while after a short time (few days) the vegetated bacilli themselves disappear. Attention is called to the observation that the tetanus spores remain in an intact state for many days in the inoculated region of the body.

The next question which I asked myself is, ***What becomes eventually of the ungerminated spores, and, supposing they remain locally in the tissue, do they suffer any alteration in toxin-producing power?***

Experiment. Rat No. 9. Inoculated June 8. Eosin-treated. No symptoms, June 24. Removed the spore-thread which was found to be surrounded by fibrous tissue and adherent to the fascia. Eosin had disappeared. Cover slips showed many spores and no bacilli. A pure culture of *B. tetani* obtained in tissue bouillon.

Rat No. 10. The thread was removed nineteen days after inoculation. The result was in all respects like that of rat No. 9. Toxin of the usual strength was yielded by the cultures.

Rat No. 11. A part of the spore-thread was removed twenty-three days after the inoculation. Cover slips showed many spores which yielded a pure culture of *B. tetani* containing a strong toxin. The second wound healed without giving rise to tetanus. Animal remained well.

By these observations it is shown that the healing of the local wound containing the eosinized threads, proceeds in a manner similar to that of a sterile wound; and it is also shown that, by virtue of the eosin treatment, the tetanus spores are reduced to the value of inocuous foreign bodies. In the course of this process, the tetanus spores, already quiescent, remain in the healed tissues for an indefinite time. They are not devitalized by the tissues, or, apparently, altered in any essential way, at least, their toxin-producing power is not impaired by the new conditions under which they survive.

If the results obtained in vitro and in vivo are compared certain correspondences, as well as certain differences, can be noted. Perhaps the most important difference consists in the disappearance of the vegetative bacilli in the body, and their persistence in the test tubes, in the tests with eosin; and the germination of the re-

strained spores in the culture once the eosin is no longer present, while, in the body, the tissues already advanced in healing restrain the vegetative propensities of the spores, although the toxin has been entirely eliminated.

The healing of the wound of the second operation in rat No. 11, without giving rise to tetanus, all the eosin having meanwhile disappeared, is an interesting fact, and one that deserves further consideration. The cultivation experiment proved that living tetanus spores, capable of toxin-production remained in the thread; and yet the remainder of the thread, in the tissues, gave rise to no symptoms. It might be thought indeed, that the spore threads, after this long sojourn in the body, are not capable of directly infecting a second animal. A test of this possibility was easily made.

Experiment. Rat No. 12. A normal rat was inoculated on July 9 beneath the skin of the thigh with a spore-thread removed on the thirty-second day from an eosin-treated rat. On July 11 the animal was tetanic, and on July 13 it was dead. A pure culture of *B. tetani* was recovered from the thread.

The apparent innocuousness of the second operation for the removal of spore-threads in animals treated long before with eosin, and the great susceptibility of the normal rat to the threads after this long residence in the body, brought up the question of the possible existence of a local immunity to tetanus in the eosin-treated animals. This idea was, of course, capable of experimental verification. The experiments relating to it form the subject of a separate publication.

SUMMARY.

Eosin, if present in cultures containing tetanus spores, prevents the germination of these spores when its concentration (in glucose bouillon) reaches 0.2 per cent. When the concentration of the eosin sinks to 0.01 per cent., germination of the spores is no longer inhibited, but the vegetative bacilli developed from the spores execute a highly restrained form of multiplication. When the eosin concentration sinks to 0.001 per cent., vegetation and multiplication of the bacilli become more active, but no new spores are formed even after long periods of time. With glucose agar it is not until the concentration of the eosin in the cultures falls to 0.05 per cent. that sporulation again appears. At this concentration of the eosin,

very few spores are formed; but as the eosin sinks lower and lower, sporulation becomes more active, until with 0.001 per cent. it is essentially of normal degree. In concentrations of 0.003 per cent., eosin prevents perfect segmentation of the multiplying bacilli, with the result that, finally, long and convoluted threads of bacilli are produced. The spores which are formed in a medium containing 0.01 per cent. of eosin are often situated at the centre and not at one pole of the bacilli.

Eosin in a strength of 2 per cent. is capable of destroying the vegetative bacilli, if the contact is prolonged to fifteen minutes, and in strength of 0.1 per cent., in twenty-four hours. Placing this latter mixture of bacilli and eosin in the sunlight greatly hastens the bactericidal effect, and the bacilli are found to be incapable of growth at the end of several hours. Eosin in high concentrations is not capable of killing the tetanus spores, even after long exposure to sunlight (thirty hours).

The toxin production of tetanus bacilli grown in eosinized culture media diminishes as the concentration of the eosin increases. This effect is brought about partly by the restraining action of the dye on vegetation, and partly by its detoxicating action upon the poison.

The toxin-producing power and the virulence of tetanus bacilli are not permanently modified by contact with eosin for a long period, or by successive cultivations in eosinized media.

Eosin is likewise capable of restraining the vegetation of tetanus spores in the animal body. In spore threads inserted beneath the skin of rats, and surrounded with eosin in solution, a very restricted vegetation takes place. If the injections of eosin are repeated, vegetation soon ceases and the vegetated bacilli degenerate and disappear.

The ungerminated tetanus spores remain alive in a latent condition indefinitely in the healed wound beneath the skin. These spores do not lose power to grow outside the body, or inside the body of animals under favorable conditions, or to produce toxin in a characteristic manner.

LOCAL IMMUNITY TO TETANUS IN INOCULATED RATS TREATED WITH EOSIN.

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Previous studies having shown the mechanism of the immunity to tetanus which develops in rats treated locally with eosin, my attention was directed by Dr. Flexner to certain indications of the occurrence in the treated animals of a local in contrast to a general immunity to the tetanus bacillus. Our studies[1] had already shown that the spores on silk threads in the healed eosin-treated wounds remain alive for an indefinite period, and the threads removed and transplanted to other rats, or to the opposite side of the body of the same rat, caused fatal tetanus. And yet, the operation for the removal of part of the spore-threads was not followed, in spite of the trauma inflicted and the portion of the spore-thread which remained, by any tentanus in the extremity originally infected. Since it appeared that the conditions were, after the second operation, similar on both sides of the body, or in the two animals, an explanation of the different reactions observed was required. It seemed natural to assume, for the sake of further experiment, that the restricted germination and vegetation which take place in the eosin-treated animals produce a small quantity of tetanus-toxin, which, acting locally upon the tissues, gives rise in them to a degree of immunity to the action of the tetanus poison.[2]

The observations upon which this hypothesis was first based will be given; but it should be stated in advance that the tetanus-bearing threads were introduced subcutaneously in the thigh and not deeply into the tissues. After the healing process is complete, the threads come to lie in a small mass of scar tissue between the superficial

[1] Flexner and Noguchi, *Jour. of Exper. Med.*, 1906, viii, 1.

[2] Noguchi, "The Nature of the Antitetanic Action of Eosin," this number of the JOURNAL.

fascia and the skin. Except for the cicatrix the tissues all return apparently to the normal condition.

Experiment I.—In order to determine whether the spores remained alive in the inoculated rats after the healing process was complete, a portion of a thread was removed thirty days after inoculation and fifteen days after the cessation of the eosin treatment; no trace of the eosin remained at this time in the tissues. The removed portion of the thread was used for preparing cultures and for inoculating rat No. 12. Tetanus bacilli grew in the cultures. Tetanus developed in rat No. 12 on the second day, and death from tetanus took place on the fourth day after inoculation. The treated and operated rat remained well.

Before discussing further the significance of this experiment, a selected series of protocols bearing on this question of local immunity to tetanus will be first given.

Experiment II. Rat No. 13.—Thirty days after inoculation and fifteen days after cessation of eosin treatment, a portion of the healed-in spore-thread was removed from the right thigh. No tetanus followed the trauma, and the wound quickly healed. Six days later, a second portion of the thread was removed and implanted subcutaneously under the skin of the left thigh. Tetanus developed on the left (new) side on the second day, and death occurred on the fourth day after this inoculation. The right (original) hind leg remained entirely free from tetanus.

Experiment III. Rat No. 14.—Eosin treatment. Seventeen days after inoculation the thread was removed from the right thigh; the wound of the second operation was completely healed in seven days, during which period no tetanus developed. July 12, reinoculation of spore-threads beneath the skin of both thighs. July 16, left leg tetanic; right leg free. July 18, tetanus has progressed on left side; right side free. July 19, rat dead. The animal lived three days after the first appearance of tetanus. The right (old inoculated) leg remained free from tetanus until the end.

Experiment IV. Rat No. 15.—Spore-thread inoculation into right leg June 8; eosin treatment until June 20. No tetanus. Spore-thread removed July 28; no tetanus. October 9 (123 days after inoculation, and 111 days after last injection of eosin), 0.00008 c.c. tetanus toxin injected into each thigh (0.0003 = m.l.d. in three days). Tetanus developed in the left leg in forty hours, and advanced rapidly; slight tetanus developed in the right leg after three days, and advanced slowly. Death on seventh day.

Experiment V. Rat No. 16.—Spore-thread inoculation into right leg October 29; eosin rein treatment until November 8. No tetanus. December 1 (33 days after inoculation and 25 days after last injection of eosin) 0.0004 c.c. tetanus toxin injected into each thigh. Tetanus developed in left leg after three days, and advanced rapidly; a mild form of tetanus developed in the right leg and advanced slowly. Both sides became strongly contracted; death on sixteenth day.

Experiment VI. Rat No. 17.—Spore-thread inoculation into right thigh October 19. Eosin gelb injection until November 2. No tetanus developed. December 1 (43 days after inoculation and 29 days after last injection of eosin),

0.0004 c.c. of tetanus toxin injected into each thigh. No tetanus developed in five days. Second injection into each thigh of 0.0004 c.c. toxin. Next day tetanus began in left leg; none in right leg. The tetanus advanced in the left and remained absent from the right side until death, which took place on the ninth day.

Experiment VI brings out clearly, for the first time, the existence of a degree of general immunity to the toxin which is, however, able to off-set a small and limited amount only of the effects of the toxin. The local immunity of the right thigh proved, upon a second injection of the toxin, to be still effective, while the general immunity, through which the left leg had been protected from the consequences of the first injection, was exhausted by it. The next experiment indicates that even a higher degree of general immunity may arise from the local infection.

Experiment VII. Rat No. 18.—Spore-thread inoculation into right thigh; eosin treatment. No tetanus developed. October 9 (123 days after inoculation and 111 days after last injection of easin), 0.00008 c.c. tetanus toxin (0.00008 = m.l.d.) injected into each thigh. No tetanus after three days. October 12, 0.00004 c.c. toxin injected into each leg. October 19, no tetanus developed. The spore-thread from this animal transplanted to another rat produced typical tetanic symptoms, followed by death in two and a half days.

Experiment VII is a stronger confirmation even than experiment VI of the idea that general immunity to tetanus toxin may be developed in the eosin-treated rats. The degree of general immunity is, in almost all the rats, lower than that of the local immunity. Rat No. 18 seems to supply the one exception to what appeared as the rule in this respect. It was now desirable to ascertain by a direct experiment whether the blood of the eosin-treated rats contained a measurable quantity of tetanus antitoxin.

Three series of rats were taken: (1) normal rats as controls; (2) rats which after the spore-thread insertion had been treated with eosin "gelb;" (3) rats which after inoculation had been treated with eosin "rein." The rats were bled from the carotid artery, and the serum collected. On account of the small amount of blood yielded by these animals, the blood of the several animals of each series was collected together. The eosin-treated rats were bled from sixty-four to seventy-four days after the cessation of the eosin treatment. The following tabulation gives the results of the tests. In each instance, the mixtures of serum and tetanus

toxin were kept at 37° C. for half an hour before injection. The injections were made beneath the skin of the thigh.

Serum Injected.	Toxin Injected.	Tetanus Developed.	Result.
Normal 0.3 c.c.	0.006 c.c. (6 m.l.d.)	24 hours.	Death on 3d day.
Normal 0.3 c.c.	0.002 c.c. (2 m.l.d.)	24 hours.	Death on 6th day.
Saline 0.3 c.c.	0.006 c.c. (6 m.l.d.)	24 hours.	Death on 3d day.
Saline 0.3 c.c.	0.002 c.c. (2 m.l.d.)	24 hours.	Death on 5th day.
Eosin gelb "a" 0.3 c.c.	0.006 c.c. (6 m.l.d.)	slightly in 48 hours.	Tetanus increased but did not extend beyond injected leg. 28 days after injection still alive; tetanus gradually disappearing.
Eosin gelb "b" 0.3 c.c.	0.006 c.c. (6 m.l.d.)	slightly in 48 hours.	Tetanus progressed, death on 9th day.
Eosin rein 0.3 c.c.	0.006 c.c. (6 m.l.d.)	slightly in 48 hours.	Death on 10th day.
Eosin rein 0.3 c.c.	0.002 c.c. (2 m.l.d.)	5th day.	Death on 15th day.

The experiments which I have set down in this article seem to me to afford convincing proof of the existence of local immunity to tetanus infection and tetanus intoxication. The observation is, I believe, new and not without interest in its bearing on the theory of the "building sites" of immune bodies in the animal organism. Examinations of the lesion consisting of the scar enclosing the spore-threads have shown it to be superficial, limited to the fascia and adjacent subcutaneous tissue, and to measure a few millimeters in extent only. There are no anatomical changes discoverable which would connect the lesion with the deeper muscles, fascia, nerves, etc.

From the studies made of the spore-threads at different periods during the eosin treatment,[8] a complete idea of the evolutions through which the introduced spores pass has been obtained. A certain small number of the spores germinate and grow into vegetative bacilli, which, after having reached this stage of development, remain as such for a time, or, possibly, pass through a small series of divisions with the production of a few generations of bacilli. The number of divisions is at best few, and the total number of bacilli produced from the spores is relatively small. The vegetative bacilli degenerate within a few days, and the majority of

[8] Noguchi, "The Nature of the Antitetanic Action of Eosin," this number of the JOURNAL.

the spores do not germinate at all, but remaining alive and quiescent, are enclosed in the scar tissue.

Doubtless, there is associated with the low degree of vegetation of the tetanus bacilli toxin production. This toxin, always small in amount, is held chiefly in or near the locality in which it is formed by the surrounding wall of eosin through which it must, in the first place, pass slowly and with difficulty, and which, in the next place, would tend to detoxicate it on its passage. The effect, therefore, of the toxin would be exerted locally, if anywhere, and if any cells capable of being excited to antibody-production exist in the situation in which the toxin finds itself, they, presumably would be stimulated into activity. Should any of the toxin, in a still active state, diffuse beyond the eosin barrier, it would find itself in places from which it could readily pass into the blood and into the internal organs, where it would be exposed to the usual conditions of antibody formation. I conceive that the mechanism of the antibody production in the particular case brought out by these experiments is to be explained somewhat in the manner of this supposition.

The questions immediately arise as to which cells in the local tissues are concerned with the elaboration of the antitoxin, and whether the antoxin as such is stored in these cells for immediate liberation when called forth by the presence of the specific antigen. As regards the first question, no definitive answer can be given. From the circumstances of the experiment, I am led to suppose the connective tissue cells, possibly the endothelium of the lymphatics as well, as the cells yielding the antibody. The extremely superficial character of the lesions must be kept in mind in attempting to fix the parts the cells of which participate actively in the production of this local immunity. But I cannot bring forth proof that the active cells are so strictly limited and so sharply circumscribed as I have indicated. It would, theoretically, seem to be less difficult to supply an answer to the second question. It does not seem to me highly likely that so diffusible a substance as antitoxin in the free state could remain in a state of high concentration through many months in one part of the body at the same time that it existed in very low concentration elsewhere in the body.

On the contrary, I should be more inclined to the view that a large part of the general antitoxic immunity which the rats exhibited may have been derived from the antibody absorbed from its local site of production. The cells of the locally immune tissues have, I assume, undergone a physiological change which endures for many months, at least, enabling them to withstand the injurious effects of tetanus bacilli and tetanus toxin either by means of rapid liberation of antitoxic substances, or by an increased form of resistance to and destruction of poison (Giftfestigkeit) and bacilli with which the liberation of antagonistic antibodies is not necessarily associated. Upon what remarkable changes in function or structure this power depends, we are in total ignorance, and any speculation must, therefore be wholly hypothetical.

The literature on immunity contains, as is well-known, other examples of local immunity. Doubtless in every case, at some period of antibody production, there exists a local immunity which exceeds in degree that which the blood is able to display. This fact must be acknowledged by all who believe antibodies not to be formed chiefly in the blood itself. It will suffice merely to refer to the observations of Pfeiffer and Marx[4] on the organs in which the antibodies to the cholera bacillus are produced; to the experiments of Wassermann and Takaki[5] of the fixation of tetanus toxin by the brain and other organic tissues; to the studies of Wassermann and Citron[6] on the local production by the pleural and peritoneal endothelium of antibodies (bacteriolytic, possibly others also) for the typhoid bacillus; to the interesting experiment of von Dungern[7] on the local production of precipitin for Maja serum in the anterior chamber of the eye; and, finally, to the observations of opthalmologists[8] upon the local immunity developed by the conjunctiva to abrin inoculation, a phenomenon which Römer studied with great care and precision. Theoretically, there is much similarity in all these observations, since they prove that anti-

[4] *Zeit. f. Hygiene und Infektionskrank.*, 1898, xxvii, 272.

[5] *Berl. klin. Woch.*, 1898, xxxv, 5.

[6] *Zeit. f. Hygiene und Infektionskrank.*, 1905, l, 331.

[7] Die Antikorper, Jena, 1903.

[8] Von Hippel, *Archiv f. Ophthalmolog.*, 1883, xxix, 213. Sattler, *Klin. Monatsbl. f. Augenheilkr.*, 1883, xxi, 207. Neisser, *Fortsch. d. Medicine*, 1884, ii, 73. Römer, *Archiv f. Opthalmologie*, 1901, lii, 172.

bodies are produced locally. The gradually increasing number of facts relating to this subject tend to exalt in importance cells which hitherto have been regarded as indifferent in respect to antibody production: namely, the cells of the connective tissues, lymph spaces, lymph vessels and serous cavities. The particular observations which form the basis of this paper show that the antibodies to the tetanus poison can be produced in quantity by other cells than those of the central nervous system, for which, apparently, tetanus toxin has an especial affinity.

PHYSIOLOGICAL AND PHARMACOLOGICAL STUDIES OF MAGNESIUM SALTS.[1]

V. The Influence of Nephrectomy upon their Toxicity.

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Introductory.—The narcotic effect as well as the toxicity of magnesium salts, when introduced subcutaneously, depend to a great degree upon the readiness with which they are eliminated from the body. By what path are these salts eliminated? Are they excreted by the intestinal canal or are they eliminated through the kidneys? Meltzer and Auer[2] state that from their experiments they gained the impression " that animals which urinated frequently had a better chance for recovery. Early urination seemed also to prevent the full development of anesthesia. Furthermore it seems that magnesium sulphate after subcutaneous injection acted as a diuretic." Such observations seemed to indicate that the kidneys are taking an essential share in the elimination of the magnesium salts. On the other hand J. B. MacCallum[3] states that magnesium salts (magnesium chloride) not only do not act as diuretics, but exert directly an inhibitory effect upon the urinary secretion. Furthermore from a remark recently made by Mendel it seems to follow that magnesium as an alkali earth leaves the body by the bowel. Mendel[4] states that the studies of the paths of excretion for inorganic compounds seem to " indicate a noteworthy difference in the mode of elimination of these alkali metals in contrast with corresponding salts of the alkali earths. The latter have been shown to leave the body in far greater quantity by the bowel than in the urine."

[1] Studies I–IV were published in the *American Journal of Physiology.*

[2] Meltzer and Auer, *American Journal of Physiology,* 1905, xiv, 386.

[3] MacCallum, " On the Mechanism of the Physiological Action of the Cathartics," Berkeley, 1906, p. 45.

[4] Mendel and Closson, *American Jour. of Physiol.,* 1906, xvi, 45.

Of the alkali earths Mendel himself studied the elimination of strontium[5] and barium salts.[6] For the paths of elimination of calcium salts Mendel refers to Rey.[7] For the salts of these three alkali earths it was variously established experimentally, as stated above, that no matter by what path introduced, they leave the body in far greater quantity by the bowel than in the urine. Magnesium like calcium is an alkali earth and from the rule laid down by Mendel for this class of inorganic compounds it seems to follow that the salts of magnesium also leave the body in far greater quantity by the bowel than in the urine. Are there any experimental facts for such an assumption?

Mendel who does not discuss specially the paths of elimination of magnesium, gives only one reference; it is to Lusk.[8] But here we meet with a statement which is contrary to that assumption. Lusk says that "in carnivorous urine the major part of excreted magnesium is found in the urine, the balance being given off through the intestinal wall to the feces." Lusk mentions no authority for his statement. But the literature on metabolism of inorganic salts contains many studies which bear out this view of Lusk. In the extensive studies of F. Müller,[9] for instance, we find a statement which is exactly like the one made by Lusk. He says "dass beim Fleischfresser der Kalk zum weitaus grössten Theil durch den Darmkanal aus dem Organismus ausgeschieden wird, während die Magnesia . . . hauptsächlich durch den Harn verlässt." Other writers speak of the fraction of magnesium salts which leaves the body through the kidney as being between thirty and forty per cent. However this may be, there is a unanimity of opinion amongst all the investigators, at least as far as we can see, on one point which is of especial interest to us, namely that there is a striking difference between calcium and magnesium with reference to the path of elimination,

[5] Mendel and Thatcher, *American Jour. of Physiol.*, 1904, xi, 5.

[6] Mendel and Sicher, *ibid.*, 1906, xvi, 147.

[7] Rey, *Archiv f. exp. Path. u. Pharm.*, 1895, xxxv, 295. See Rüdell, *ibid.*, 1893, xxxiii, 79.

[8] Lusk, "American Text-Book of Physiology," 2d edition, 1900, Vol. i, p. 527.

[9] Müller, *Zeit für Biol.*, 1884, xx, 334. Heiss, *ibid.*, 1876, xii, 156. Bertram, *ibid.*, 1878, xiv, 335. Renvall, *Skandinavisches Arch. für Physiol.*, 1904, xvi, 94.

the former preferring the alimentary canal, while the latter favors the kidney. In the quoted metabolism experiments the salts of magnesium and calcium were administered by the mouth. As far as we know, there are no experiments on the mode of elimination of magnesium salts when they were given subcutaneously or intravenously. Possibly in the latter cases the elimination through the kidney would prove to be still more favored. At any rate the rule laid down for the elimination of calcium, strontium and barium cannot be applied *a priori* to the elimination of magnesium.

We may therefore claim that there is, to say the least, no experimental evidence based on chemical analysis against the assumption that the kidneys are taking an important share in the elimination of magnesium salts. On the other hand, the above quoted observation of Meltzer and Auer "that animals which urinated frequently had a better chance for recovery" may be taken as an indication in favor of such an assumption. The authors, however, had at that time not made a special study of that question; they speak only of their impressions. Besides, the fact that urination and recovery went hand in hand is not safe evidence that the urination contributed to the recovery. Possibly the recovery takes place through the elimination of magnesium into the intestines and urination occurs on account of the removal of magnesium and in accordance with the above mentioned claim of MacCallum that the presence of magnesium in the blood inhibits urinary secretion; the coincidence of recovery and urination would then mean that recovery is the cause and urination the result and not the contrary.

There is, however, another biological method by which the share of the kidneys in the elimination of the magnesium salts can be systematically studied and which is capable of bringing out reliable results. It is the study of the susceptibility of the animal to the toxic effect of magnesium salts after nephrectomy. Such a series of experiments were carried out by us and the object of this paper is the report of the findings of this research.

THE SUSCEPTIBILITY OF THE ANIMAL TO THE TOXIC EFFECT OF MAGNESIUM AFTER NEPHRECTOMY.

Method.—The experiments of Meltzer and Auer on the anesthetic effect of subcutaneous injection of magnesium salts were made on seven species of animals and with the sulphates as well as with the chlorides. The present experiments were made on rabbits only, and only magnesium sulphate was employed. Under ether anesthesia double nephrectomy was performed and after recovery from the ether anesthesia variable quantities of magnesium sulphate in molecular solution were injected subcutaneously, using approximately the same region for injection in all animals and observing the same conditions. The results of this research will be presented best by quoting a few abbreviated protocols of experiments.

Fatal Dose.—In the following experiment one gram of the salt per kilo animal was injected shortly after nephrectomy.

Experiment 1.—June 19, 1906. A series of three rabbits was used.

A. White rabbit, 2,480 grams. 3 p. m. Both kidneys exposed by lumbar route, pedicles firmly ligated and the greatest part of each kidney removed.

3.35. Rabbit recovered from ether, appears quiet and normal. Injected magnesium sulphate ($\frac{M}{1}$ solution), *one gram per kilo weight.*

4.10. Animal drowsy, chin rests on floor, head to one side, respiration shallow.

4.35. Lying on side, completely relaxed.

4.43. No conjunctival reflex; reacts feebly to probe in nose.

5.05. Respiration very shallow, 36 per minute.

5.15. Respiration stopped, heart still continues to beat. Animal died one hour and forty minutes after the injection.

B. (Nephrectomized control.) Black rabbit, 2,340 grams; appears to be less robust than A.

3.20. Nephrectomized as in A.

4.00. Does not recover from the anesthesia as rapidly as A; trembles (carbolic intoxication?).

4.20. Hops around, rises on hind legs and seems normal.

June 20. Eats cabbage, appears normal.

June 21, 10 a. m. Did not eat at morning feeding, drinks a little water.

12 noon, found dead.

This animal died about 45 hours after nephrectomy.

C. (Magnesium control.) White and black rabbit, 1,625 grams, not operated.

3.30. Injected magnesium sulphate ($\frac{M}{1}$ solution) one gram per kilo weight.

At no time was there the slightest sign of a magnesium effect, the rabbit appearing at all times as normal as before the injection.

In this series one gram of magnesium sulphate per kilo animal weight killed the nephrectomized animal one hour and forty minutes after the injection (two hours and fifteen minutes after the operation), whereas the same dose had not the slightest effect upon the normal rabbit and the nephrectomized control animal which received no magnesium, lived forty-three hours and appeared to be normal until a few hours before its death. From the experiments of Meltzer and Auer it appears that a dose less than 1.75 grams per kilo, when given subcutaneously and without massage is not fatal to the rabbit. For the nephrectomized rabbits a dose of only one gram of the salts per kilo animal proved to be fatal; that means an increase of the susceptibility of the animal to the poisonous effect of the magnesium salt nearly equal to fifty per cent.

Experiment 2.—Series of two rabbits. June 25, 1906.

A. Gray rabbit, 2,500 grams.

2.15 p. m. Double nephrectomy.

4.10. Injected one gram magnesium sulphate per kilo animal.

4.30. Animal shows signs of beginning anesthesia and paralysis. All symptoms gradually increased and the animal died at 7 p. m. about three hours after the injection.

B. Control (Rabbit C from former experiment). Not operated.

June 25, 4.10 p. m. Injected 1.6 magnesium sulphate per kilo animal.

5.30. Animal greatly relaxed, lid reflex much retarded. Anesthesia and paralysis were at no time complete; the animal soon began to show recovery, and next morning appeared to be perfectly well again.

In this experiment again in the nephrectomized rabbit one gram per kilo killed the animal in a few hours after the injection, while in the normal animal 1.6 grams per kilo was even insufficient to produce complete temporary anesthesia.

In these and in other similar experiments it was then found that one gram per kilo, injected two or three hours after nephrectomy, proved to be a fatal dose, the animal becoming first anesthetized and paralyzed a few hours after the injection.

Effect of Late Injection.—The result was different, however, when the injection was not administered until about eighteen hours or more after the nephrectomy. The following protocols will illustrate.

Experiment 3.—June 26, 1906. Rabbit A, weighing 1,992 grams, was nephrectomized at 6.30 p. m. Rabbit B, weighing 1,957 grams, was nephrectomized at 6.50 p. m.

A. June 27, 12.35 p. m. (eighteen hours after nephrectomy). Injected magnesium sulphate one gram per kilo in $\frac{M}{1}$ solution; *slight massage.*

2. Chin rests on floor, animal remains lying on side when turned over; recovers posture when tail is pressed.

4.45. Animal lying stretched out, relaxed, conjunctival reflex slight. Reacts to probe in nose and on pressing tail.

June 28, 11.40 a. m. Animal shows some recovery, occasionally makes an effort to rise; lid reflex retarded, but definitely present; on pressing the tail, the animal moves head and feet and cries feebly.

Animal died at 1.30 p. m., twenty-five hours after injection, forty-three hours after operation.

B. June 27, 12.40 p. m. About eighteen hours after nephrectomy injected magnesium sulphate one gram per kilo in $\frac{M}{1}$ solution; massage, stronger than in A.

1.20. Strongly influenced by the salt. Lies flat, head on floor. Raises head when tail is pinched, lid reflex still present.

4.40. Lid reflex completely gone; no reaction to pressure of tail or to probe in nose.

Animal died at 8 p. m., about seven hours after the injection, twenty-five after nephrectomy.

Both animals were injected about eighteen hours after nephrectomy and in both the injected places were massaged, in *B* a good deal more strongly than in *A*. Animal *A* was never completely under anesthesia, had shown later some slight recovery and lived about twenty-five hours after the injection. Animal *B* was completely under anesthesia and died without recovery about seven hours after the injection.

This last rabbit (*B* of Experiment 3) was the only animal which lived so short a time as seven hours after the injection when the latter was given late after nephrectomy. Some animals lived twenty-four hours and more after the injection, and one rabbit lived as long as sixty-three hours after an injection of one gram of the salts per kilo animal, which was given twenty-four hours after the double nephrectomy. The narcotizing effect was in this case fairly outspoken and the recovery was very slight, the animal remaining for about three days in the semi-paralyzed and semi-stuporous state.

In general it may be stated that the later after the nephrectomy one gram salt per kilo was given, the less fatal it proved to be and also, perhaps, the less complete was the state of depression; the recovery, however, was in all cases but slight.

Anesthetic Dose.—In eight experiments the dose of the salt injected into the rabbits amounted only to 0.8 gram per kilo animal. We shall illustrate the results by one abbreviated protocol.

Experiment 4.—June 21, 1906. Gray rabbit, 1,520 grams.

1 p. m. Double nephrectomy.

1.50. Injected magnesium sulphate 0.6 grams per kilo in $\frac{M}{1}$ solution.

3.50. Lying flat, completely relaxed, respiration very slow. Conjunctival reflex present, but diminished.

4.45. Lying completely relaxed on side, conjunctival reflex gone.

6. No change.

June 22, 1.30 p. m. Improved; able to sit up; conjunctival reflex returned.

June 23, 12 noon. Rabbit died, about forty-six hours after injection.

In this experiment with 0.8 grams per kilo, injected very soon after nephrectomy, the animal was completely anesthetized, the depression lasting about twelve hours followed by a moderate degree of recovery. The animal survived the injection by forty-six hours, death apparently being due solely to the nephrectomy. In other experiments with the same dose and under the same conditions the profound anesthesia lasted sometimes more than twenty

FIG. 1. All four rabbits were nephrectomized about twenty-two hours before the photograph was taken. The two animals lying on the table received two hours after the nephrectomy subcutaneous injections of magnesium sulphate, 0.8 per kilo, and about two hours later were completely under the toxic influence of that salt. They remained for about eighteen hours profoundly anesthetized and paralyzed. At the time when the photograph was taken the rabbit on the right hand side showed a slight recovery. The other two rabbits are (nephrectomized) controls.

hours; in other cases again the anesthesia was sometimes a little less complete and the recovery a trifle better than in the last quoted experiment (see Figure). But in the entire series there was no case without distinct anesthetizing and paralyzing effect and none with a fatal outcome due to the magnesium salts. It is hardly necessary to add that in normal rabbits a dose of 0.8 grams per kilo had not the slightest effect.

In a few experiments a dose of 0.8 grams of the salt per kilo animal was given twenty-four hours after nephrectomy. The following protocol is an illustration.

Experiment 5.—July 2, 1906. Grey rabbit, 1,810 grams, 11.40 a. m. Double nephrectomy, perfect recovery.

July 3, 1.54 p. m., about twenty-six hours after nephrectomy, injected magnesium sulphate 0.8 grams per kilo in $\frac{M}{1}$ solution.

3.00 p. m. Animal relaxed, lid reflex very slight.

4.00. Lid reflex gone; no spontaneous movements and no reaction to pressing tail, etc.

10.00. Condition unchanged.

July 4, 10 a. m. Slightly improved, moves head when probe is put in nose, but no reaction to pinching of tail and no lid reflex.

July 5, 10 a. m. Considerably improved; can almost sit up; lid reflex retarded, but present; moves away on pressing tail.

3 p. m. Recovery still more pronounced. Head erect; movements irregular and jerky.

July 6, 8.30 a. m. Found in a dyspnœic state; the animal died five minutes later, about sixty-seven hours after the injection and about ninety-three hours after the operation.

This animal, although injected twenty-six hours after the nephrectomy, became influenced by the salt very rapidly; the depression was very profound and lasted over twenty-four hours. It survived the injection, however, longer than any other animal and the final recovery was more advanced.

Here again, as in the experiments in which a dose of 0.8 grams of the salt was given soon after the nephrectomy, the extent of the effect of the injection was somewhat variable, but the effect was on the one hand never missing and on the other hand never fatal.

In general it may be said that for a dose of 0.8 gram per kilo of the salt there was not a marked difference between the effects of the injections whether given soon after nephrectomy or twenty-four hours later, and this difference certainly cannot be compared

with the striking differences observed in the experiments with one gram per kilo animal.

Minimum Dose.—We have made seven experiments in which a dose of only 0.6 gram of the magnesium salt per kilo rabbit was injected. The following few abbreviated protocols will illustrate the corresponding results.

Experiment 6.—June 21, 1906. Gray rabbit, 1,520 grams.

1 p. m. Double nephrectomy. Completely recovered.

3.20. Injected 0.6 gram of magnesium sulphate per kilo animal in $\frac{M}{1}$ solution

4.45 and 6 p. m. Appears normal in every way. Animal remained normal until its death which occurred June 24, 8 p. m., about seventy-two hours after injection and seventy-four hours after operation.

In this animal 0.6 gram per kilo had apparently no effect whatsoever.

Experiment 7.—July 6. White rabbit, 1,610 grams.

11 a. m. Double nephrectomy; complete recovery.

2 p. m. Injected 0.6 gram magnesium sulphate per kilo in $\frac{M}{1}$ solution.

3.15. Some loss of muscular control, especially of hind legs; reflex much retarded.

5.15. Stretched out, completely relaxed; lid reflex sluggish but present; reacts promptly to pinch of tail.

8.15. Shows some recovery; able to sit up.

July 7, 9.30 a. m. No further recovery. General muscular weakness, especially of the anterior part, reacts sluggishly to stimulation, lid reflex retarded. Animal remained in the same state until it died July 8, 7 p. m. It lived fifty-three hours after the injection and fifty-six hours after the operation.

This animal was moderately but distinctly influenced by a dose of 0.6 gram per kilo. It recovered from a major part of the depressing influence within five hours after the injection, but retained some degree of depression until death which was apparently due solely to the nephrectomy.

These two experiments represent both extremes of the results. Out of seven experiments with 0.6 per kilo three have shown practically no signs of depression. In the other four animals anesthesia and paralysis were present in a variable degree, but were never complete and lasted only a few hours. In two the recovery was not complete.

In two experiments the injection of 0.6 gram per kilo was given about twenty-four hours after the nephrectomy. In one the de-

pression was hardly noticeable and in the other the depression was well marked, but of only short duration with complete recovery.

Apparently in nephrectomized rabbits a dose of 0.6 gram per kilo is bordering on the minimum toxic dose and the various uncontrollable minor factors which frequently influence to a slight extent the degree of absorption cause this dose to be sometimes without any effect and at other times to produce a well defined, though only temporary influence.

Cumulative Effect.—An interesting point is the question of the cumulative effect. In normal animals the effect of several subminimum doses administered at various times is not equal to the effect of the sum of these doses when given in single injection on account of the elimination which takes place during the intervals between the injections. It was different, however, with the behavior of magnesium salts in nephrectomized animals, as can be seen in the following experiment.

Experiment 8.—July 6, 1906. White rabbit, 1,740 grams.
12 noon. Double nephrectomy.
2.15 p. m. Injected magnesium sulphate 0.3 gram per kilo in $\frac{M}{1}$ solution.
3.30 and 4 p. m. Animal normal.
4.15. Injected again, 0.3 gram per kilo of the magnesium salt.
4.30. Animal lying on side, completely under anesthesia; lid reflex gone.
8.15. Seems somewhat improved; moves head.
8.20. Injected again 0.3 gram per kilo.
11 p. m. Animal profoundly under anesthesia.
July 7. 9 a. m. Somewhat improved; moves head and feet, but lid reflex still absent.
9.25. Injected again (fourth time) 0.3 gram per kilo.
12 noon. Profoundly under anesthesia; respiration very shallow.
Animal died at 2 p. m.

In this animal two subminimum doses of 0.3 gram per kilo given two hours apart had after the second dose at least as much effect as 0.6 gram per kilo given in one dose. A similar result was obtained when a dose of 0.2 gram per kilo was given hourly. About half an hour after the third dose the animal became fairly well anesthetized and recovered again after a few hours. In these cases, if the interval between the injections did not exceed two or three hours, the cumulative effect was perfect and in fact the sum of two or more doses seemed to be even more effective than a cor-

responding single dose. Possibly the cumulative effect might have been due to the fact that a single dose was given in only one place, while the several smaller doses were given in several different places, the latter circumstance favoring absorption.[10]

Discussion.—The following facts were brought out by the foregoing series of experiments.

A dose of one gram magnesium sulphate per kilo animal, when injected subcutaneously within two or three hours after double nephrectomy proved invariably to cause death a few hours after injection. In normal animals such a constant result could be obtained only with a dose of 2 grams per kilo.

A dose of 0.8 gram per kilo, injected within two or three hours after nephrectomy, was never fatal to the animal, but the animal became invariably more or less completely anesthetized and paralyzed. In normal animals such a constant effect could be attained only with a dose not less than 1.6 gram per kilo.

The susceptibility to the anesthetic and toxic effects of the magnesium salts was therefore in the nephrectomized animals increased with about fifty per cent. This increase of susceptibility is apparently due to the decrease in the facility of elimination of the salts by the urine. These experiments therefore tend to show that normally the kidneys carry off at least fifty per cent. of the injected salts. We should, however, lay little stress upon the exact figures. But we are, we believe, justified in stating that the experiments demonstrate conclusively *that the kidneys play an essential part in the elimination of the magnesium salts.*

In harmony with this conclusion is the observation which was made upon the cumulative action of the magnesium salt in the nephrectomised animals. The effect of the two or three injections of subminimum doses was at least equal to the sum of these quantities given in a single dose; a fact which can only be explained by the assumption that during the intervals none of the salt was eliminated. This observation would seem to contain the suggestion that at least during the first few hours after an injection no elimination takes place except through the kidneys. We shall, however, not dwell too much on this side of the question.

[10] Meltzer, *Jour. of Exper. Med.*, 1901, v, 643.

A further instructive fact is the observation that the profound anesthesia and paralysis produced by an injection of a dose of 0.8 gram per kilo lasted undiminished twelve to twenty hours and sometimes a good deal longer. Furthermore the recovery which finally took place was only moderate, the animal remaining until death in a pronounced state of paresis and stupor in sharp contrast with the nephrectomized control animal. In normal animals the anesthesia which was brought on by an efficient, but not fatal dose lasted at the utmost two hours and then the recovery was complete. This fact again can be best explained by the assumption that in the nephrectomized animal no fraction of the absorbed salts is eliminated for twelve or eighteen hours.

An interesting fact finally is the observation that a dose of one gram per kilo which is invariably fatal when given soon after nephrectomy is no longer fatal when injected eighteen to twenty-four hours after nephrectomy, although it still produces deep anesthesia; in other words eighteen to twenty-four hours after nephrectomy a dose of one gram per kilo acts like a dose of 0.8 per kilo injected soon after nephrectomy. This seems to indicate that some time after nephrectomy vicarious paths of elimination develop which thus assist in converting a fatal dose into only an anesthetic dose. This assumption would also explain the cause of the recovery from deep anesthesia twelve or eighteen hours after an injection of a dose of 0.8 gram per kilo; as at that time some of the salt becomes eliminated.

The degree of this vicarious elimination, however, is apparently very small; it never converted a fatal dose or an anesthetic dose into a harmless one, and the recovery from the anesthesia is only moderate, the animal generally remaining until death under a considerable influence of the salts.

The results which were obtained by us in the study of the effect of nephrectomy upon the toxicity of magnesium salts are in marked contrast to the results obtained by Meltzer and Salant[11] in their study of the effect of nephrectomy upon the toxicity of strychnin. Strychnin is generally assumed to be eliminated essentially through the kidney. Meltzer and Salant nevertheless found that the mini-

[11] Meltzer and Salant, *Journ. of Exper. Med.*, 1905, vi, 107.

mum toxic dose is for nephrectomized rabbits the same as for normal ones. For magnesium we found that for nephrectomized rabbits the dose is half of that which is toxic for the normal animals. Furthermore, the cumulative effect of strychnin is remarkably small. In intervals of two or three hours subminimum doses can be given, until they equal the sum of two or three times the toxic dose before any toxic symptoms will appear. For magnesium we found that the toxic effect will appear as soon as the sum of the subminimum doses becomes equal to the single minimum dose which is capable of producing an effect. Finally in nephrectomized rabbits no dose of strychnin was ever observed to produce continuous convulsions for any length of time; the animals either succumb soon or the convulsions gradually subside. With magnesium we observed that in nephrectomized rabbits the anesthesia may last uniformly for twenty-four hours and longer. Strychnin apparently finds soon after nephrectomy a satisfactory vicarious path for its elimination from the body, at least in rabbits. It should be mentioned that for guinea pigs Meltzer and Langmann[12] observed that within the first three hours after nephrectomy the toxic dose is indeed smaller than for the normal animal and that at that period in some animals a subminimum dose produced a continuous vibration which the authors termed a *subtetanic reaction.* They explained these observations by the assumption that in guinea pigs during the first few hours after nephrectomy vicarious paths for the elimination of strychnin are not yet developed.

Vicarious paths for elimination of magnesium even in rabbits do not develop until late after the nephrectomy and even then only in an unsatisfactory manner.

Conclusions.—Magnesium salts when introduced subcutaneously are eliminated to a great extent through the kidneys. In nephrectomized rabbits the susceptibility to the toxic effect of magnesium salts is increased by about fifty per cent.

The profound anesthesia which a toxic dose of magnesium produces in nephrectomized rabbits may be continuous for twenty-four hours and longer.

The cumulative effect of magnesium salts in nephrectomized

[12] Meltzer and Langmann, *Journ. of Med. Research,* 1903, ix, 19.

rabbits is very striking. The effect of several subminimum doses is equal to the effect produced by the sum of these doses given in a single injection.

A dose which when given soon after the nephrectomy is fatal, causes only a non-fatal anesthesia when given eighteen hours or later after the nephrectomy. Probably at that period vicarious paths develop sufficient for elimination of a fraction of the salts.

It is, probably for the last mentioned reason, that the profound anesthesia produced by a proper dose of the magnesium salts is partially recovered from about twelve to eighteen hours after nephrectomy.

THE INFLUENCE OF SALICYLIC ACID UPON URICOLYSIS.

By L. B. STOOKEY AND MARGARET MORRIS.

(From the Laboratory of Physiology, Medical Department, University of Southern California.)

In a recent paper one[1] of us pointed out that subcutaneous injection of liver-extract (dog) might lead in the case of the dog to an increased power on the part of the liver to destroy uric acid in vitro. From these results it seems possible that the pharmacological action of some drugs might be connected with an increase in the rate of uricolysis. That the salicylates might act in this manner occurred to us as a possibility. The plausibility of this assumption relative to the action of salicylic acid seemed to be strengthened by the finding by one[2] of us that the liver, kidney, spleen and muscle taken from dogs treated with sodium salicylate showed a rate of uricocylis greater than that found in organs taken from normal dogs.

To test the accuracy of this view regarding the action of salicylates, dogs were injected subcutaneously (0.1 grm. sodium salicylate in one per cent. solution per kilo body weight) daily over periods of five and ten days, and the uricolytic power of the organs taken from the treated dogs was compared with that of organs taken from normal dogs under identical conditions. In all cases the dogs were bled to death, the organs removed as soon as possible and hashed finely, and a solution of sodium urate of known strength added to weighed portions of the macerated organs. Toluol was added to prevent bacterial action and the flasks were maintained at blood-temperature throughout the experiment. The uric acid remaining at the conclusion of the experiment was determined in duplicate by the method of Folin and Schaffer.[3] The average of the estimations is recorded.

[1] Stookey, L. B., *Jour. Med. Research*, 1906, xv, 321.

[2] Stookey, L. B., *Proc. Soc. Exper. Biol. and Med.*, 1907, iv, 59.

[3] Folin and Schaffer, *Zeit. für physiol. Chem.*, 1901, xxxii, 552.

Our results are contained in the following table.

No. of Dog.	Description of Dog.	Organ.	Grms. of Organ Taken.	Uric Acid Solution.		Length of Exposure in Hours.	Uric Acid Destroyed; in Grms.	Uric Acid Destroyed in Terms of 100 Grms. Organ in Grm.
				%	Vol. in c.c.			
I.	Normal.	Liver.	31	0.2	200	46	0.1210	0.3900
II.	Treated 10 days.	Liver.	20	0.2	150	44	0.0850	0.4250
III.	Normal.	Liver.	20	0.2	150	47	0.0743	0.3715
IV.	Treated 5 days.	Liver.	40	0.1	200	72	0.1685	0.4212
II.	Treated 10 days.	Liver.	20	0.2	150	67	0.1020	0.5100
III.	Normal.	Spleen.	11	0.1	150	47	0.0174	0.1581
II.	Treated 10 days.	Spleen.	11	0.1	150	44	0.0330	0.3000
II.	Treated 10 days.	Spleen.	9	0.1	150	67	0.0307	0.3416
I.	Normal.	Spleen.	13	0.1	150	67	0.0249	0.1921
III.	Normal.	Kidney.	27	0.1	150	46	0.0735	0.2722
II.	Treated 10 days.	Kidney.	19	0.2	150	44	0.1166	0.6138
III.	Normal.	Kidney.	27	0.1	150	70	0.0667	0.2472
IV.	Treated 5 days.	Kidney.	16	0.1	200	72	0.0750	0.4777
I.	Normal.	Kidney.	27	0.2	150	47	0.0660	0.2444
II.	Treated 10 days.	Kidney.	19	0.2	150	67	0.535	0.2815
II.	Normal.	Muscle.	20	0.1	150	47	0.0630	0.3000
II.	Treated 10 days.	Muscle.	20	0.1	150	44	0.0915	0.4575
III.	Normal.	Muscle.	65	0.1	200	46	0.0760	0.1169
III.	Normal.	Muscle.	58	0.1	200	70	0.0815	0.1404
II.	Treated 10 days.	Muscle.	20	0.1	150	67	0.0874	0.4370
I.	Normal.	Muscle.	58	0.1	200	144	0.0820	0.1413

An increased ability to destroy uric acid on the part of the kidney, muscle, spleen and liver taken from the dogs treated with sodium salicylate is noticeable.

Experiments regarding the influence of other agents upon uricolysis are in progress.

ON THE FUNCTION OF THE POSTERIOR SPINAL GANGLIA.

By S. P. KRAMER, M.D., Cincinnati.

In the course of an investigation on the nature of shock, certain phenomena concerning the influence of the spinal ganglia on the inhibition of shock were observed, and seem worthy of some special notice. In a previous publication[1] the author has described the effect upon the respiration and circulation produced by irritation of the central end of a divided sensory nerve, *i. e.*, the crural nerve, in dogs, and the variation in these effects with different degrees of anesthesia (ether).

During the experiments ether was administered through a tracheal tube. The blood pressure and cardiac contractions were recorded by means of a mercurial manometer, connected with the right carotid artery. The movements of the thorax were recorded by means of the exploring tambour of Paul Bert attached to a strap of adhesive plaster which encircled the lower portion of the chest. This tambour was connected through a rubber tube with a Marey tambour whose writing lever thus recorded the movements of expansion and contraction of the chest. The upstroke represented the inspiratory movement; the downstroke, the expiratory. The time was recorded by means of a metronome beating seconds placed in a circuit with an electrical signal, thus transmitting its rhythm to the signal, which marked the time on the paper of the kymograph.

The right crural nerve was divided and the central end stimulated electrically. For this purpose Du Bois Reymond's apparatus with platinum electrodes was used. An electric signal was placed in the primary circuit of the apparatus, so that the time during which the nerve was irritated was marked on the paper of the kymograph. Many experiments were made on the same animal in various stages of anesthesia. In only one dog was no anesthetic employed. The results here were constant and had been described before, so that I did not deem it justifiable to repeat this experiment.

[1] Kramer, *Annals of Surgery,* 1900, xxxii, 377.

Irritation of the central end of the divided crural nerve in an animal that had received no narcotic always produced the same effect. The respiration was increased in frequency about fifty per-cent. and the inspiratory effort was markedly increased. The cardiac rhythm was also increased approximately to the same extent. The blood pressure invariably rose. These effects gradually disappeared after the cessation of the stimulation, so that about one minute thereafter the respiratory rhythm and force were as before, and the same was true of the circulatory apparatus.

If the animals were deeply narcotized with ether, so that there was absolutely no corneal reflex, the result was very different. In most instances the irritation of the central end of the crural was without effect either upon the circulation or respiration.

At times there was a very slight effect on the respiration in that the inspiration was slightly inhibited. The rhythm remained unchanged and the circulation was not disturbed. There was however, a stage of ether anesthesia during which the effect was materially different. If the animal were anesthetized, but only partially, so that there was a condition of semi-anesthesia, in which the corneal reflex was present, but in which the animal lay quietly on the table, and if the cural nerve were irritated, the respiration, just as in unnarcotized animals, became greatly accelerated in rhythm and increased in amplitude. On the part of the circulatory apparatus, a very interesting change was observed. Instead of an increase in the pulse rate and a rise in pressure, as occurred in unnarcotized animals, we had a marked fall in blood pressure. This fall began a few seconds after the beginning of the irritation of the nerve, lasted for a varying number of seconds, and eventually returned to the level present before the irritation. The fall in blood pressure was greater the more intense the irritation, that is, the nearer the coils of the faradic apparatus were placed.

In every experiment there was a stage of general anesthesia in which irritation of the central end of the crural nerve produced this fall in blood pressure.

Experiments were also made upon animals narcotized with ether with a view of determining the influence of the posterior spinal ganglia on the transmission of these impulses. The posterior roots

were exposed and one of the lower dorsal roots was divided peripherally to the ganglion; the electrodes were applied to the divided posterior root both centrally and peripherally to the ganglion. It was found that the spinal ganglion had an inhibitory effect. That is, a degree of irritation, which, in a given degree of anesthesia was without effect on the circulation or respiration when applied peripherally to the ganglion, would, when applied centrally, that is between the ganglion and the spinal cord, be followed by the "vasomotor shock," described above as following irritation of a sensory nerve during incomplete anesthesia. The spinal ganglion undoubtedly holds back or inhibits the effect of this irritation on its way to the cord and brain.

Respiration

Circulation.

Period of irritation.

TRACING I. Electrode applied peripherally to the ganglion; anesthesia incomplete; corneal reflex present. Coils of faradic apparatus 10 c.m. apart.

Tracings I and II are records of two experiments on the same dog made about a minute apart. The animal was anesthetized with ether; the corneal reflexes were present. The coils of the faradic apparatus were ten centimeters apart.

Tracing I is a record of an experiment in which the electrodes were applied to the spinal root peripherally to the ganglion. There

is a slight increase in amplitude of the respiratory movements and practically no effect upon the circulation.

Respiration.

Circulation.

Period of
irritation.

TRACING 2. Electrode applied centrally to the ganglion; anesthesia incomplete; corneal reflex present. Coils of the faradic apparatus 10 c.m. apart.

Respiration.

Circulation.

Period of
irritation.

TRACING 3. Electrode applied peripherally to the spinal ganglion; complete anesthesia; no corneal reflex. Coils of faradic apparatus 5 c.m. apart.

Tracing II is a record of an experiment in which electrodes were applied centrally to the ganglion. We have here the effect noted before, namely a marked acceleration of rhythm and increase in amplitude of the respiratory movements, and a decided fall in blood pressure. These experiments show that an amount of irritation which, when applied to the posterior roots peripherally to the ganglion had produced practically no effect, when applied centrally to the ganglion was followed by marked "vaso-motor shock." A similar result was obtained in deeply narcotized animals in which the corneal reflex was absent, and where stronger stimuli were applied.

TRACING 4. Conditions are the same as with Tracing 3. Electrode applied centrally to the spinal ganglion.

Tracings III and IV are records of two experiments on the same dog in which the electrodes have been applied peripherally and centrally to the ganglion, the coils being five centimeters apart. The tracings show the same thing as before, that is, little or no effect when the electrodes are applied peripherally and marked "vaso-motor shock" when applied centrally to the ganglion.

It is believed that the registration of these phenomena will provide a valuable method for studying the functions of the spinal ganglia under various conditions. The author intends at some future time to report the results of his researches along this line.

THE PATHOGENESIS OF GALL STONES.

By S. P. KRAMER, M.D., Cincinnati.

The ultimate cause for the formation of gall stones has, notwithstanding the large amount of investigation on the subject, remained a subject for speculation.

The earliest view was a purely mechanical one; that the formation of the stones was the result of concentration due to stagnation. On account of the fact that bile may be concentrated to a thick mass without the precipitation of the products found in gall stones, this view had to be abandoned.

There then followed the so-called chemical theory, the chief exponent of which was Thudichum.[1] Cholestearin, bilirubin and the calcium salts are soluble in alkaline solution, especially in solutions of sodium glycocholate, and concentration does not bring about precipitation. If, however, according to Thudichum, as the result of decomposition due to prolonged standing or as the result of abnormal secretion of mucus, the bile becomes acid, the cholestearin and bilirubin calcium are precipitated as the result of the decomposition of the solvent, sodium glycocholate, into glycocoll, cholalic acid and a sodium salt. In reading the work of Thudichum, it is well to remember that the work was done a half century ago, and of course, without bacterial investigation.

Another chemical explanation was offered by Dochmann[2] in 1891. He analysed both liver bile and bladder bile, the latter obtained after ligation of the cystic duct, and found a great increase in calcium and diminution in sodium in bladder bile as compared with liver bile. According to his view the increase in calcium diminishes the solubility of bilirubin and leads to a precipitation of bilirubin calcium and cholestearin. Prolonged intervals between meals and stasis cause a stagnation and precipitation of bilirubin calcium and cholestearin.

[1] Thudichum, *Quarterly Journal of the Chemical Society*, 1862, xiv, 114.

[2] Dochmann, *Wien. med. Presse*, 1891, xxxii, 1198.

Opposed to these chemical theories are the morphological and bacteriological theories. Dujardin Beaumetz[3] ascribes the formation of gall stones to a desquamative cholangitis and biliary stasis, thus reviving the "Stein bildende Katarrh" of Meckel.[4]

Naunyn[5] denies the chemical theory of Thudichum. Even when greatly concentrated, bile contains sufficient solvent to keep the cholestearin and bilirubin calcium in solution. The decomposition of sodium glycocholate to sodium cholate does not explain the precipitation, since this product is also a solvent for cholestearin and bilirubin calcium. He also ascribes the formation of gall stones to a desquamative cholangitis.

This inflammatory process leads to a desquamation of epithelial cells which degenerate and form cholestearin and calcium salts. The bilirubin unites with the calcium. The precipitation of bilirubin calcium is aided by the albuminous character of the products of cellular degeneration, alluding here to the general tendency of albumen to precipitate calcium.

In this way are formed small particles which form the nuclei of gall stones. The cholestearin is deposited later, partly on the outside and partly infiltrating the mass through the so-called infiltration canals which are found in gall stones. The mere excess of calcium will not cause a precipitation of bilirubin in calcium and cholestearin. Even the presence of foreign bodies as centers of crystallization will not bring this about.

There is lacking the explanation of some biological process which brings this about, and this, according to Naunyn, is furnished by the degeneration of epithelial cells the result of an infective cholangitis.

We have still, however, no explanation as to the process by which cholestearin and calcium salts, which are normally soluble in bile, are precipitated in cholelithiasis. To say that it is the result of degenerative changes in epithelium, produces more mystery, but no explanation.

If now we leave the realm of speculation and search for some exact knowledge, a very curious chain of thought will arise. We

[3] Dujardin Beaumetz, *Bul. Therap.*, 1891, cxxi, 291.
[4] Meckel von Helmsbach, "Mikro-geologie," 1856, Berlin.
[5] Naunyn, "Klinik der Cholelithiasis," 1892.

have, heretofore, exact knowledge of the formation of stones in one instance and in one only. I refer to the formation of phosphatic urinary calculi. We know that this is due to chemical decomposition, the direct result of bacterial growth. If now we apply this knowledge to experiments, having for their object the clearing up of the subject of gall stones, it may be that some success will follow. It has been abundantly proven, that bacteria are at all times present in gall stones. The literature on the subject is so well known that it is not necessary to burden this article with it.

The colon bacillus is the organism most frequently found in gall stones. The one next in frequency is the typhoid bacillus. It is very probable that it will be found that infection by one of these two micro-organisms is responsible for most cases of cholelithiasis.

Now while this has been commonly accepted, it seems strange that there should be no investigation published, showing the effect of these organisms on bile when grown in it.

It would appear *a priori* reasonable, that possibly the growth of these organisms in bile, or a solution of bile, would effect the precipitation of some of the biliary constituents. Accordingly, for the past year I have been carrying out such an investigation with most gratifying results.

Culture tubes were prepared containing a mixture of one half human bile obtained at autopsy and one half ordinary alkaline pepton bouillon. This mixture or solution was repeatedly sterilized and filtered, until a perfectly clear medium was obtained. Such tubes were inoculated with the colon bacillus, the typhoid bacillus and Staphylococcus pyogenes aureus. All of these micro-organisms grow readily in this medium. The staphylococcus forms a copious growth which sinks to the bottom of the tube, the medium showing no apparent change.

Not so, however, with those inoculated with the colon or typhoid bacillus. Here in a few days the medium became cloudy and a precipitate is seen at the bottom of the tube. This precipitate increases greatly until in about four weeks a very well marked, closely packed, semi-solid mass is seen at the bottom of the tube. If allowed to incubate longer, say for six months, care being taken to prevent evaporation by rubber caps, this precipitated mass becomes

firmly packed and the super-natant fluid may be poured off and we have a kind of very soft "gall stone" as it were, taking the form of the bottom of the tube. If we examine the precipitate, we have no difficulty in recognizing all the constituents of gall-stones as well as masses of bacilli: Amorphous calcium phosphate, magnesium phosphate, calcium carbonate, biliary coloring matter and a few crystals of cholestearin. Crystals of ammonio-magnesium phosphate are formed very late, usually after many weeks of growth.

In order that the precipitation of cholestearin might be made more manifest, and since, normally, bile contains comparatively

FIG. 1. Photo-micrograph of precipitate in culture of *Bacillus coli*, in medium containing 50 per cent. bile and 1 per cent. cholesterin. Culture sixty days old.

little of it, culture tubes were prepared in which cholestearin was added to the solution of bile and bouillon. This readily dissolves therein and the medium was carefully filtered and examined microscopically before inoculated, so as to be sure that all the cholestearin present was in solution. Such tubes when inoculated with

colon or typhoid bacillus, give a precipitate very rich in cholestearin crystals, as may be seen from Fig. 1, a photomicrograph of such a precipitate.

Another thing which was remarked, was the markedly preservative action of bile upon colon and typhoid bacilli. I have at present tubes in which the precipitate was separated and allowed to dry out completely, and yet the bacilli present are still viable and readily resume growth when inoculated upon fresh media. This corresponds with the findings that viable typhoid bacilli have been found in the interior of gall stones years after the individual had passed through the attack of typhoid fever.

Thus Droba[6] reports a case in which he obtained the typhoid bacillus from gall stones removed seventeen years after the patient had recovered from typhoid fever.

It appears to me that these experiments indicate very clearly then that gall stone formation, just as phosphatic urinary stone formation, is due to a chemical decomposition of the bile, the direct result of the growth of micro-organism therein. Bacillus coli communis, and Bacillus typhosus are the micro-organisms usually concerned. Just what the exact chemical nature of the decomposition is, remains to be shown. It is rather significant, however, that the two micro-organisms which cause this precipitation in vitro, produce an acid reaction in the media. Whereas, the staphlococcus, which requires and retains in akaline reaction, does not cause the precipitation.

It may be then that the decomposition theory of Thudichum will prove to be true in a modified form. His work on the decomposition of bile was done, of course, before the days of pure culture, and was without bacterial control. A repetition of his analyses of bile decomposed by pure culture of these organisms, may give us the exact chemical nature of the process.

[6] Droba, *Wien. klin. Woch.*, 1899, xii, 1141.

MULTIPLE MYELOMA: A HISTOLOGICAL COMPARISON OF SIX CASES.[1]

By HENRY A. CHRISTIAN, M.D., Boston.

Plates IX–XII.

The salient features of the clinical history and pathological anatomy of multiple myeloma are at the present time very generally recognized and need not be reiterated in this place. The finer histology, especially the nature of the cells composing the tumor, is still a matter of discussion. The variety of opinions existing as to the nature of myeloma cells is probably a result of the infrequency of the condition. Few observers have had the opportunity of comparing material from several cases similarly hardened and stained. Case reports are fairly numerous, however, as the disease is not excessively rare. Still it is difficult to judge of the exact relations of cells when they can be compared only from verbal description, aided by some form of pictorial illustration. For this reason it has seemed to me worth while to compare side by side sections from several cases, especially as no previous observer has done this.

Through the courtesy of several friends[2] I have been able to study Zenker hardened material from six cases of myeloma, some of which are reported here for the first time. All tissue was embedded in paraffin and for general study, sections were stained with eosin and methylene blue, phosphotungstic acid hematoxylin, and Mallory's anilin blue connective tissue stain.

Case I (H. M. S., 2148) was autopsied by me 4½ years after laminectomy. This operation was done by Dr. J. C. Munro of Boston, and the case then reported by Dr. John J. Thomas of Boston (*Boston Med. and Surg. Jour.*, 1901, CXLV, 367).

At the time of the operation, December 20, 1900, the patient, a man of thirty-

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[1] Received for publication May 18, 1907.

[2] To all these, as well as to others who aided in the search for material, I take great pleasure in expressing my thanks.

nine years, gave the following history.[8] About August 15, 1900, he began to feel severe pain in the back between the shoulders. This confined him to bed for four or five days at this time and since then continued but in not severe enough form to prevent his being up and about. In October he began to notice slight uncertainty in the use of his legs; his feet were numb; there was a sensation of tightness around his abdomen; he had slight disturbance of micturition. Physical examination on October 16th showed slight swaying in Romberg position, superficial reflexes absent, deep reflexes normal, sensation to touch and pain diminished below the level of the spine of the 8th dorsal vertebra. There was a slight kyphosis of the spine, more marked in the upper dorsal region. On November 16th slight weakness of the leg muscles was apparent but no atrophy was present. The knee jerks were now symmetrically increased in activity and a double ankle clonus was present. Pain and temperature sensations were much diminshed below the level of the 4th dorsal vertebra, sensation to touch slightly decreased. On December 17th the gait was slightly spastic-ataxic. His legs showed marked paresis. Now a swelling of the left 5th rib, not tender on pressure, was noticed. On December 20th laminectomy was done by Dr. J. C. Munro and the 1st to 4th dorsal laminæ were removed. The medulla of the spinous process and lamina of 4th dorsal vertebra was found to consist of soft reddish material which could be traced to the head of the rib and body of the vertebra. As much of this as possible was curetted away. Histological examination showed it to be myeloma.

Urinary examinations in December and subsequently showed the constant presence of Bence Jones' albumose.

After operation the patient developed complete paraplegia with retention of urine but this gradually improved and the wound healed well. On January 5, 1901, all movements of legs and feet could be performed normally and with good strength and there was marked improvement in sensations. After leaving the hospital the lump on the left rib became larger and then diminished in size; occasionally it was painful and tender and in March distinct crepitance could be elicited over it. During May a well marked swelling along the left 6th and 7th ribs was noted. The tenth dorsal spine was tender, and the spinal column was held rigid. At this time the patient moved only with difficulty though he could stand without support.

March 6, 1905, Dr. Thomas made the following note. Patient has been up and about most of the time. There have been occasional transient periods of tenderness over rib or vertebra. About Christmas time he had an attack of vomiting and diarrhea after eating fish. After this he was much weaker. Recently there has been pain and tenderness over the right acromion and numbness on the ulnar side of the right arm. There is a kyphosis of the upper thoracic region of 110° to 120° and considerable limitation of motion of the head. There are several swellings over ribs, but these are not tender.

Soon after this the patient had an attack of pneumonia, and died March 14, 1905.

Autopsy at Quincy, Mass., 12 hours post mortem. A well developed, fairly well nourished man, 155 cm. long. Moderate degree of rigor mortis is present

[8] This part of the clinical history was abstracted from the paper of Thomas (*loc. cit.*).

and there is slight livor of dependent parts. Cutaneous surfaces are pale, not jaundiced. Over the dorsal vertebræ near the median line, high up, is a scar of an old operation wound. In the right deltoid region there is a slight swelling having a somewhat soft feel. In the right anterior axillary line an irregularity can be felt in the 7th rib (old fracture). No very definite nodules can be felt in the ribs or sternum.

The dome of the diaphragm reaches to the 4th intercostal space on each side. The muscles of thorax are pale red, firm, and moderately developed. Left pleural cavity contains about 300 c.c. of a slightly turbid, yellowish fluid in which float numerous large flocculi of fibrin. The right is free from fluid and adhesions. Pericardial cavity contains about 75 c.c. of clear straw-yellow fluid. Serous surfaces smooth and glistening.

The heart appears slightly larger than normal for a man of this size. It contains chicken fat and cruor clots; not distended. Pericardium normal. Myocardium firm, pale red, appears normal. Valves normal. Endocardium shows here and there a small area of increased opacity, otherwise normal. Coronaries normal.

The surface of the left lung is extensively covered with a thick shaggy layer of easily removed, grayish fibrin. Lower lobe is solid, flabby, and dark red in color. Upper lobe is more voluminous, paler, especially anteriorly and everywhere air containing. On section lower lobe is red, slightly moist, finely granular and solidified; upper lobe is œdematous, and much fluid containing fine air bubbles escapes from its cut surface. This œdematous condition is most marked in lower part of lobe, and is scarcely present along anterior border.

Right lung has a smooth glistening surface, is fairly voluminous and air-containing for the most part. Posterior parts are much darker red than the anterior. In the upper lobe near its center can be felt an ill-defined, irregular area of consolidation about 3 cm. in greatest diameter. No consolidation in the middle and lower lobes. On section the upper lobe is grayish pink, œdematous and in the center irregularly consolidated. In this latter part the color is light gray, slightly granular. The cut surface of the lobe is dark red, smooth, quite dry, and from it can be squeezed a considerable amount of dark blood. Lungs throughout show moderate anthracosis. Bronchi contain a considerable amount of muco-pus; their mucous membrane is slightly congested. Peribronchial lymph nodes are slightly enlarged.

Abdominal panniculus adiposus is about 2 cm. thick. Peritoneal cavity is free from fluid, surfaces smooth and glistening. Vermiform appendix lies in the right iliac fossa curled on itself behind cœcum, and in part lies extraperitoneal. There are no adhesions about it. Mesenteric and retroperitoneal lymph nodes are not enlarged.

Spleen, wt. 210 gms., is pale, grayish red, soft and flabby, tearing easily as it is removed. On section it is almost diffluent, light grayish brown, with markings indistinguishable. Stomach contains a considerable amount of fluid. Mucosa is normal. Intestines contain very little material. They are quite friable but show no lesions. Pancreas is firm; appears normal.

Liver is distinctly tough, and is somewhat enlarged. Color brown; surface smooth. Here and there are irregular areas of marked pallor extending a varying distance into viscus, never sharply demarcated. Cut surface of organ

except for these is uniformly brown and markings are very indistinct. Gall bladder is filled with bile; no calculi.

Kidneys, wt. 280 gms., capsule strips readily from a smooth surface. Cortex measures 5 mm. in average thickness. Cut surface is very pale gray and opaque. Here and there in the cortex are small foci of congestion. Scattered irregularly in the cortex are small pin point areas of an opaque, almost white, color. Glomeruli are just visible. Striations of pyramids indistinct. No amyloid reaction with Lugol's solution.

Adrenal glands normal. Bladder normal. Prostate not enlarged. Testes normal.

Aorta normal. No tumor nodules found in abdominal or thoracic viscera.

Sternum anteriorly presents no abnormalities. Posteriorly the mid-portion of the manubrium shows a slight swelling of rounded contour the center of which is distinctly soft. Here a knife cuts readily through thin bone into a very soft dark red tissue in which very few spicules of bone can be detected. Other parts of sternum present no nodules or soft areas.

Right 7th rib shows on external surface the line of a healed fracture with imperfect apposition of ends. On pleural surface the right 3rd and 5th ribs near the anterior axillary line present distinct swellings, soft to touch, covered by a smooth pleura containing small injected vessels. On pleural side of 6th rib there is a pale irregular bony exostosis suggesting point of old fracture. On left side in same general region the 3rd, 4th, 5th, and 6th ribs show similar soft nodules, and on the 8th there is a small bony spine approximately 5 mm. in length. A rib with soft nodules was removed from each side. A description of these will suffice as other soft nodules incised in situ showed little variation. The rib was split for its entire length along the middle of its flat surface by means of a band saw. At the point of situation of the soft nodule described above, the peripheral zone of dense bone is reduced to a thin layer not continuous over entire surface but absent over a small area (Plate IX, Fig. 1). At this point the rib measures 1.2 cm. in thickness, and but for this thin layer of bone consists of a soft moist tissue, dark red in color, in which bone cannot be felt. At another point at some distance from this nodule is another area similar in color but where the rib is not so much increased in diameter. Close inspection of each area shows that these nodules are not separated sharply from adjacent marrow but gradually shade off into a tissue, likewise soft but paler, mottled gray, grayish pink or yellowish gray in color which replaces entirely the marrow of the bone. Everywhere there is but a thin (1–2 mm.) peripheral zone of bone, though from this bone spicules extend to a certain degree into the central part of the shaft. The other rib does not show such definite nodules but at several points on its surface feels soft. On splitting, its entire center is seen to be replaced by a soft tissue of varying color lying within a cortical zone of bone 0.5–2 mm. thick. In this are a few spicules of bone, but the tissue for the most part is soft. Areas 1–2 cm. in greatest diameter are dark red, other parts are gray or pinkish gray. These ribs fracture easily on manipulation. On each side in the region of attachment of the 5th, 6th, 7th, and 8th ribs to vertebræ in the angle between ribs and spinal column, are scattered nodules, soft, covered by injected pleura, rounding out this angle though not encroaching to any extent on the cavity of the thorax. Section of these nodules show them to be

soft, apparently not containing bone. Most of these are dark red, often resembling a blood clot. Some have a narrow grayish, peripheral zone, while others are reddish gray throughout. As far as seen by section through their superficial portion they are discrete but appear to arise from bone beneath.

Opposite the attachments of 6th, 7th, and 8th ribs the spinal column is bent backward and to the right to form a rather sharp curve. The superior surface of the bodies of the vertebræ from third dorsal to third lumbar were sawn away. The central parts of the bodies of all of these vertebræ are softer than normal. The center of those in the region of curvature is very soft and the marrow is semidiffluent, dark grayish red. The others, not so soft, are lighter, grayish red to gray, but for the most part irregularly mottled.

Examination of right femur shows dense cortical bone and pale yellowish marrow.

Incision of soft area over right shoulder shows beneath deltoid muscle and entirely above and unconnected with head of humerus or acromion a gray-white, quite friable material, having a greasy, soapy feel, and apparently little structure.

Anatomical Diagnoses: Multiple myeloma (ribs, sternum, and vertebrae); curvature of spinal column; old fractures of ribs; lobar pneumonia (left lower lobe); bronchopneumonia (right upper lobe); pulmonary oedema and congestion (right and left upper lobes); fibrinopurulent pleuritis (left); acute splenic tumor; chronic bursitis (subdeltoid bursa); healed scar of laminectomy.

Microscopic Examination: Heart muscle is normal and there is no connective tissue increase. Lung: All the alveoli are filled with an exudate of serum containing numerous cells. The greater part of the cellular portion of the exudate consists of polynuclear leucocytes. There is a moderate number of mononuclear cells with and without ingested polynuclear leucocytes. Some of the latter, especially those without inclusions, show irregular processes as if a cell in amoeboid motion had been fixed in that position. The alveolar epithelium is swollen and occasionally these cells are seen in mitosis. In one place a cell was fixed during its passages through the alveolar wall and a portion of the cell can be seen in each alveolus with a narrow connecting bar of cytoplasm. Peribronchial lymph node shows moderate anthracosis, and fibrosis.

Liver: Cells about the center of lobules show moderate bile pigmentation. In the cytoplasm of many of these cells appear one to several dots staining intensely blue black with phosphotungstic acid hematoxylin. About many of these there is a palely staining zone three or four times the diameter of the dot. Pancreas normal.

Spleen shows a few groups of rather coarse, bright yellow, pigment granules. Kidney shows many small irregular areas of connective tissue proliferation. This can usually be traced in from the capsule and is fairly cellular. Here and there are sclerosed glomeruli and tubules. In the pyramids many hyaline casts are seen. Most of the tubular epithelium and glomeruli appear to be normal.

Adrenal: Many of the cells in the medullary portion are pigmented. Testis normal. Tissue from subdeltoid bursa is almost entirely necrotic; in a few places there are small foci of lymphoid cells and fibroblasts.

Bones: Of the bones examined, ribs, sternum, vertebrae and femur, all showed macroscopic evidence of extensive involvement with the exception of the femur. There is a striking uniformity in the sections taken from different bones, and the structure of the tumor is very simple. The tumor is very cellular and consists of cells varying only moderately in size, shape and structure (Plate IX, Fig. 2). The cells lie close together in a coarse meshed reticulum, usually of very fine fibrils. In a few places the reticulum is coarser, more prominent and where this is the case there is rather more connective tissue in association with the larger blood vessels, but nowhere does the stroma dominate and in most places it is extremely slight. In connection with the stroma there are a few cells of the fibroblast type. In the tumor are numerous blood vessels, often of large calibre but almost always thin walled, usually hardly more than an endothelial layer with a few fibrils of connective tissue between it and the tumor cells. Scattered among the tumor cells are many red blood corpuscles and large areas of hæmorrhage.

The tumor cells have no definite spacial relations but are scattered loosely in the meshes of the reticulum, now separated so adjacent cells hardly touch, now more crowded so that the cells press each other into polygonal shapes. Here and there a row of cells is crowded into a crevice between coarser fibrils and the cells assume a cuboidal shape. Neither the phosphotungstic acid hematoxylin nor anilin blue connective tissue stains demonstrate any fibrils closely related to the tumor cells and the stroma appears to arise from the accompanying cells of the fibroblast type.

The individual cells (Plate IX, Fig. 3) are round, oval, roughly polygonal or cuboidal, dependent upon the degree of crowding as above described. There is no cell membrane and not infrequently the cell edge is slightly ragged. The cells vary in size from 8 to 20 microns. The average of the measurements of a number of cells such as are most numerous in the sections is 11.4 x 7.7 microns for cell body and 8.1 x 7.2 for nucleus. The cytoplasm is slightly basophilic as shown in the sections stained by the eosin and methylene blue method when the differentiation is not carried very far. The blue stain is, however, quite easily removed by the alcohol before the nuclei show much if any dimming of the brilliancy of their blue. Still compared with the fibrous tissue and cytoplasm of fibroblasts or epithelial cells this basophilic reaction is distinct. The cytoplasm is finely granular but granules in the Ehrlich sense were not apparent in sections stained in the usual ways and could not be demonstrated with Giemsa's stain in the method of Schridde (*Centralbl. f. allg. Path.*, etc., 1905, xvi, 769) or with Wright's modification of the Romanowsky stain used in the way described by Wright for the demonstration of blood platelets in tissues (*Boston Med. and Surg. Jour.*, 1906, clv, 30). In some of the cells the cytoplasm has in part a finely vacuolated appearance and where this condition occurs near the edge of the cell, that border is apt to be quite ragged. The staining reaction of the cytoplasm of a given cell is almost always uniform throughout and the distinct clear zone described near the nucleus in several cases is not present.

Most of the nuclei (Plate IX, Fig. 3) are round or oval. They have a distinct nuclear membrane, are vesicular and have a fine chromatin network with coarser chromatin masses attached to the nuclear membrane and scattered throughout the central part of the nucleus. However these chromatin masses are not large enough or numerous enough to give a very darkly staining nucleus. In the center lies a distinct round, homogeneous slightly refractile body 1½ to 2 microns in diameter which stains pink in eosin and methylene blue preparations and in modified Romanowsky stains (Giemsa, Wright). In phosphotungstic acid hematoxylin preparations it has a reddish brown color. This affinity for acid stains is easily demonstrated and such a body is entitled to be called a nucleolus.

Many of the nuclei when single have a slightly eccentric position in the cell (Plate IX, Fig. 3). Cells with two nuclei are frequent and those with three or four are not uncommon. Cells with several nuclei are the larger forms, 14–20 microns. In some of these the nuclei are still connected by a thread of nuclear material. The structure of the cells with multiple nuclei is the same as that described for mononuclear cells. A few nuclei have irregular shapes either horse-shoe or with a rounded bud. Occasionally one is dumb-bell shaped. No mitotic figures could be found in a careful study of many sections. While irregular nuclear shapes are seen, few of these suggest amitosis, and no evidence of division of the nucleolus can be found.

Besides the cells described, others, considered by me as degeneration forms, occur. These are cells whose cytoplasm is denser, homogeneous and acidophilic. The nucleus is shrunken and stains deeply though somewhat irregularly. Not infrequently the nucleus is fragmented. All gradations between these cells and unchanged tumor cells can be found. These cells have a slightly yellowish tinge in parts of some preparations and with many stains closely resemble nucleated red blood corpuscles. This similarity is greatly heightened in the regions of hæmorrhage where the cells have definitely taken up hæmoglobin. However a study of these cells in many sections stained in a variety of ways has convinced me that they are degenerated cells and not an evidence of an erythroblastic function as claimed by Ribbert (*Centralbl. f. allg. Path.*, etc., 1904, xv, 337) for the cells of the tumor which he reports.

In preparations stained with phosphotungstic acid hematoxylin and properly differentiated, many of the tumor cells are seen to contain in the cytoplasm near the nucleus a pair of small, round, blue black granules; sometimes only one can be seen. No other similar granules occur in the cytoplasm. In cells with more than one nucleus a group of three or more of these granules can often be found. These dots are considered to be centrosomes.

In sections where tumor and bone marrow abut, no sharp line of separation is present but tumor cells are found intermingled with normal bone marrow elements and fat cells. Even at some distance from tumor nodules scattered cells identical in appearance

with the tumor cells are found in otherwise normal looking marrow. This condition is best seen in the marrow from the femur which in the gross appeared normal and surrounded by dense unchanged cortical bone. Nevertheless in the femur marrow are definite tumor nodules.

In many sections the trabeculæ of bone have been in part or completely destroyed by the growth of tumor. The cortical bone of the ribs has in places completely disappeared but for the most part the tumor remains within the limits of the periosteum. In places however muscle and fat in the neighborhood of the tumor is invaded and groups of tumor cells are found between muscle fibres or fat cells.

Tissues about the tumor show slight inflammatory reaction indicated by invasion with lymphoid and a few plasma cells. In places in the fibrous tissue are many connective tissue cells filled with coarse yellow pigment granules. The same pigment is sometimes found within tumor cells but this is unusual and there is no other evidence of phagocytosis on the part of the tumor cells. Scattered here and there in the tumor are small foci of lymphoid cells. Much of the tumor shows very extensive hæmorrhage and this is responsible for the deep red color macroscopically evident.

Case II (J. H. H., 1666) was autopsied by Dr. W. G. MacCallum at the Johns Hopkins Hospital. The clinical notes of this case were published by Hamburger (*Johns Hopkins Hospital Bull.*, 1901, xii, 38) from whom I quote a brief extract. The patient was a negress of 50 admitted to the Johns Hopkins Hospital October 10, 1900. For a year she had had pain in the region of the right groin and hip. Last June while picking up a bucket of coal she experienced a remarkable sensation of lengthening in the left arm, and next morning she found that she could not raise this arm to her head because of pain. About a week later the right arm was somewhat similarly affected. About this time she noticed a swelling the size of a hen's egg on the back of her head. Six days before coming to the hospital her right leg gave way while walking, and since then she has been unable to stand.

In the hospital a fluctuating mass 10 cm. in diameter was found in the occipital region. This was not adherent to the skin, not movable on the deep tissues and not tender. Over the inner third of either clavicle was a nodule 3 to 4 cm. in diameter, and in the left supraspinous region another 4 cm. in diameter. Upper third of thigh was markedly enlarged by a tumor the size of a child's head. The patient's urine contained Bence-Jones' albumose. She died February 1, 1901.

At autopsy MacCallum (*Jour. of Exper. Med.*, 1901, vi, 53) found tumor masses

in femur, ilium, clavicle, sternum, scapula and skull. There were no metastases in abdominal or thoracic viscera. He described the tumors as follows: "These growths presented everywhere the same appearance. Everywhere they evidently sprang from the marrow of the bone from which they were not by any means sharply demarcated. . . . The well defined tumor masses were perhaps somewhat firmer than such a mass of bone marrow would be. They varied somewhat in consistence, however. In general they were soft; some of the larger were almost diffluent and flattened out when cut and laid on the pan. Others were less soft and in some parts the gelatinous pulpy consistence gave way to a fair degree of firmness. In color there was also considerable variation. The greater part of the masses was of a deep red color, perhaps even darker than normal bone marrow but showing everywhere a grayish tint. Usually lines and streaks of gray were to be seen throughout this deep red, and in nearly all the masses definite nodules of firmer consistence and of a grayish white color were found. At some points there was a slight yellow opacity."[4]

Microscopic Examination.[5]—The tumor is made up almost entirely of cells (Plate IX, Fig. 4). Connective tissue reticulum is very slight in amount and the connective tissue fibrils appear to have no intimate association with tumor cells. Where the connective tissue is larger in amount there are narrow bands and strands enclosing coarse meshes or spaces filled with tumor cells. Tumor cells appear to have no definite spacial relations; here they are closely crowded together, there more widely separated. Blood vessels are present in moderate number and are relatively thin walled and of large calibre. About the blood vessels are a few connective tissue fibrils, some of which radiate out among the tumor cells. However, between individual cells fibrils of connective tissue are rare.

The cells (Plate IX, Fig. 5) themselves vary somewhat in shape according to the degree to which they are crowded together. Usually they are rather polygonal with one diameter greater than the other. When separated from one another they are round or oval. The average of the measurements of a number of the cells occurring most abundantly in the tumor is 10.4 x 6.8 microns. Other cells however occur which are considerably larger, some with several nuclei, others with a single quite large nucleus. Smaller

[4] For further details of this case see papers of Hamburger and MacCallum.

[5] This description has been made by me independently of that given by Dr. MacCallum (*loc. cit.*). It has been made on preparations prepared in the same way as the preceding case. The same is true for the other cases which have been elsewhere described.

apparently shrunken cells also are found. Yet the prevailing cell picture is one of a quite uniform size.

The cell outline is sharp but no cell membrane is evident. In eosin and methylene blue preparations the cytoplasm has a very finely granular ground glass appearance or is homogeneous. Some cells have a finely vacuolated cytoplasm and not infrequently these cells may have a ragged outline due to vacuolization of their peripheral portions. The cells are slightly basophilic. Many of the cells have a yellowish hue, even in unstained preparations. This is particularly evident in hæmorrhagic portions and suggests hæmoglobin staining. When this is associated with certain forms of nuclear degeneration to be described later, there is much resemblance to erythroblasts.

In phosphotungstic acid hematoxylin stains the cytoplasm appears distinctly more granular. In many cells near the nucleus there is a rounded pale zone surrounding one or two small dots staining deeply blue which I regard as centrosomes. In some cells especially those with several nuclei three or more such centrosomes may be seen grouped together. Some of the large cells are phagocytic and contain cells, usually red blood corpuscles, in varying stages of disintegration, or yellow pigment masses.

Nuclei (Plate IX, Fig. 5) are round, sometimes oval, sometimes slightly irregular in contour with pseudopods or indentations. They are almost always eccentrically placed in the cell. Some of the larger cells show nuclear parts connected with one another by a very fine thread of nuclear material. The average of a number of measurements of nuclei is 6 x 6 microns. Nuclear structure perhaps can be described most clearly by giving a description of one form and noting the modifications from this type that occur. However it is to be understood that the nucleus described first is not the one that is found in the majority of the cells but instead the first modification is more frequent. The nucleus to begin with is vesicular. It has a distinct cell membrane slightly and irregularly thickened by small chromatin masses. In its center is ordinarily a rounded metachromatic mass slightly smaller than that in the tumor cells of Case I. This is considered a nucleolus. Between nucleolus and nuclear membrane are a few very fine chromatin threads and granules.

The most common variation from this nuclear structure and the one seen in most cells of the tumor seems to arise in this way. There is a moderate increase in chromatin. Most of this chromatin tends to a peripheral position against the nuclear membrane and in the form of larger chromatin masses. Some of these masses however may remain free from the nuclear membrane. Nucleolus may still be evident. With this change there is a very slight decrease in the size of the nucleus. In a few cells chromatin clumping continues and the central portion of the nucleus almost entirely loses its chromatin. The end result of this change may be a nuclear membrane with 3 to 5 large chromatin masses adherent to it, and the central portion of the nucleus free from stainable substance. Next the nuclear membrane disappears, the chromatin masses become round and there results a cell with 2 to 5 small intensely staining separate masses of nuclear material.

In other nuclei this end stage appears to have been attained in a different way. First the chromatin becomes arranged in what appears to be a coarse twisted thread very similar to the coarse skein stage of karyokinesis but with the difference that along the threads chromatin gathers in the form of rounded swellings. This thread lies in a vacuole and all suggestion of a nuclear membrane is lost. There is no granular change in the cytoplasm such as accompanies true cell division. As the change progresses apparently the chromatin masses grow larger, the connecting thread smaller until finally complete separation results.

A third form of nuclear change is a simple shrinkage with condensation of the chromatin. This results in a small round solidly staining nucleus. The later stages of these three forms of nuclear change when associated with hemoglobin staining of the cytoplasm give cell pictures almost identical with those frequently seen in the megaloblasts and normoblasts of pernicious anemia.

True indirect cell division occurs but karyokinetic figures are difficult to find. However some cells were seen which showed nuclear figures with the associated granular changes in the cytoplasm and the loss of sharp outline of the cell body. Frequently, however, it is impossible to determine whether a cell nucleus is undergoing division or some form of degeneration.

Scattered here and there are cells of the same general size as the predominating cell, or a little larger, with a somwhat different nucleus in that the structure is irregular, slightly indented, more deeply staining because it contains larger chromatin masses more closely aggregated together. Sometimes these cells have two nuclei. These cells as well as the tumor cells first described differ quite distinctly in appearance from the elongated connective tissue cells which accompany the stroma.

In sections somewhat differently fixed, the cells show very much more frequently and more distinctly the round, pinkish nucleolus which was seen in some of the cells in the other section.

In another section, probably bone marrow, there are islands of cells, 40 or 50 in number, scattered amid loose fat tissue. Here almost all the cells are round, but the nucleus preserves its tendency to an eccentric position. Otherwise the cells show much the same characteristics as in other sections.

As in Case I there is a slight inflammatory reaction about the tumor masses and similar pigmentation.

Case III (H. M. S., 2318) was a man, a patient of Dr. John C. Munro, of Boston, who was admitted to the Carney Hospital for the first time on November 10, 1904.[*] He was at that time 49 years old. Eleven months prior to this, suddenly he was seized with an attack of pain localized in the back in a small area at the level of the spine of the scapula and just to the left of the spinous processes of the dorsal vertebræ. This pain was almost continuous, and after nine months was accompanied by pain in the left arm, sharp and constant, which radiated to the painful area in the back and was worse on motion of the arm. There was also tenderness in these regions. For two months his arm had been wasting and growing weaker. This was accompanied by difficulty in flexing fingers and inability to touch thumb to any but index finger. Four months ago he noticed three hard lumps on top of his head, which gradually increased in size. Two months ago he noticed a similar hard lump on one of the ribs of the left side. None of these were painful or tender.

When admitted to the Carney Hospital there were three swellings on his head and two in left chest wall. These were not tender. There was pain on moving head and in back on left side at level of scapula. Slight diminution in sensation to pin point was present on left side down to level of 7th dorsal spine and in left arm, especially on ulnar side, with muscular atrophy more marked below level of elbow.

On November 21st Dr. Munro removed 6th and 7th cervical and 1st dorsal laminæ. One lamina was found to be eroded and involved in a gelatinous growth

[*] The following brief history was abstracted from the clinical records of the Carney Hospital.

which extended laterally and anteriorly into adjacent structures but did not involve the dura beyond simply pressing upon it. This growth was curetted away as far as possible and the wound drained. After this symptoms were considerably improved and patient left the hospital December 5, 1904.

On May 5, 1905, patient returned with pain and tenderness over lower lumbar vertebræ. Dr. Munro performed laminectomy on 3rd and 4th dorsal vertebræ, but no tumor growth was evident. The patient left the hospital June 1, 1905.

Later the patient returned to the hospital because the tumors on head and arm were increasing in size. In the region of the anterior fontanelle there was a hemispherical, semi-fluctuant tumor the size of a half orange, and near the posterior fontanelle one as large as a walnut. In the right axilla there was a non-sensitive movable tumor the size of an English walnut, and on the right upper arm two smaller tumor masses. On January 6, 1906, Dr. Munro dissected the axillary lymph nodes and removed the tumor masses from the arm. Of the latter one was encapsulated, the other infiltrating; both appeared to be outside the periosteum. The larger tumor of the head was curetted. Both tables of the skull were found eroded and the dura pressed upon though not involved. The patient improved and left the hospital January 20, 1906. During this last stay in the hospital albumose was found in his urine. Tissue from this last operation was sent to the pathological department of Harvard Medical School and this was available to me for histological study. It was described as follows: Spherical mass from arm 2 cm. in diameter, on section soft, white, semi-opaque, homogeneous. Mass of fat from axilla, with three nodules imbedded in it, 1, 1½, and 2 cm. in diameter. On section discrete, chocolate colored, softened masses. Two much softened. Two masses from the scalp, each 20 cm. in volume, red brown in color with spicules of bone imbedded in parts, soft homogeneous, semi-translucent.

Microscopic Examination.—As in the preceding cases, the stroma is very slight in amount. However it differs somewhat from them in the fact that though the fibers are slender they form a finer meshed reticulum in which lie the tumor cells. Blood vessels are fairly numerous and relatively thin walled. The tumor is very cellular (Plate X, Fig. 6) and the cells themselves present a quite uniform appearance though varying somewhat in size. The average for a number measured is 10.5 x 6.8 microns. In shape they are round or slightly oval, somewhat polygonal where crowded together. The cell outline is distinct and regular (Plate IX, Fig. 7). In places there is some indication of a cell membrane. The cytoplasm is very finely granular, almost homogeneous. It is very slightly basophilic.

The nucleus is round or oval (Plate X, Fig. 7) usually of regular contour. Nucleus in relation to cell body is rather large. Its average size is 7.2 x 6.4 microns. The nucleus is usually slightly

eccentrically placed in the cell, but this is not a marked feature. There is a distinct nuclear membrane. The nucleus is vesicular with rather fine chromatin masses diffusely scattered. At times there is a slight peripheral grouping of the chromatin, but this is never very marked. In eosin and methylene blue preparations the chromatin granules appear finer and the nuclei more vesicular than they do in the phosphotungstic acid hematoxylin. Sometimes the chromatin is clumped towards the center and the surrounding nucleus is almost free from stainable substance. Many nuclei show a rounded metachromatic central body corresponding to a nucleolus. This, however, is much less evident than in the other cases. Still in some sections stained with eosin and methylene blue it stands out sharply and red in an otherwise blue nucleus.

A few cells have two nuclei, rarely more. These cells are larger in size. There are cells with shrunken deeply staining nuclei but these are relatively infrequent. Occasionally there is seen a cell with a nucleus as large as the neighboring cells. The nucleus of this cell stains rather deeply and the chromatin granules are coarser than in the more common cells of the tumor.

Nuclear figures are very numerous in sections of this tumor, and the cells in mitosis are distinctly granular and without a sharp cell outline. A few cells are phagocytic and contain cell remains. No hæmoglobin pigmentation of cells is seen in this case.

In a section of the lymph node from this case there are numerous areas of epithelioid cells some of which contain also giant cells and form very typical tubercles. Besides this the sinuses here and there in the lymph node are filled with cells of the identical type of those previously described as occurring in the tumor. In places it is a question whether these masses of tumor cells are in true lymphatics, or growing as masses in the structure of the lymph node itself. However, the suggestion is that the tumor is pretty well confined to the lymph channels, while in the tissue about there are lymphoid and plasma cells of the ordinary type with the possibility of an occasional tumor cell, but this latter is open to doubt.

Case IV (E. and E. I., 2–1119). Material from this case was given me by Dr. F. H. Verhoeff, of Boston. It was removed at operation by Dr. Alexander Quackenboss. The clinical notes and macroscopic description have been abstracted from the report of Quackenboss and Verhoeff (*Jour. of Med. Research,* 1906,

xv, 261). A man of 50 was admitted to the Massachusetts Charitable Eye and Ear Infirmary on November 13, 1905. Six months before this he complained of double vision and noticed a prominence of his left eye. Two months ago he noticed a swelling at the upper part of the sternum. In the Infirmary examination showed a smooth rounded tumor 6½ x 8½ cm. over the sterno-clavicular articulation. At the upper and outer half of the left orbit was a hard bone-like mass, in size and shape somewhat resembling the lachrymal gland, firmly attached to the orbit wall. Toward the nasal side another tumor is felt more elastic to the touch and situated deeper in the orbit. Portions of each tumor were removed for diagnosis. Macroscopically the tissue from both situations was soft, translucent and slightly yellow in color, somewhat resembling adipose tissue. The patient's urine contained Bence-Jones' albumin. After the operation the patient lived two months. During this time both growths increased in size and four new ones appeared over the thigh. There was no autopsy.

Microscopic Examination.—This is a very cellular tumor with sparse stroma (Plate X, Fig. 8). The stroma present is in the form of a coarse meshed reticulum enclosing many cells. Fibroglia fibrils occur but the connective tissue elements bear no close relation to the tumor cells.

The cells in pieces from the orbital and sternal tumors differ somewhat in appearance. Those (Plate X, Fig. 9) in the orbital tumor are slightly larger averaging 9.4 x 7.6 microns. They have relatively more cytoplasm and a more eccentric position of the nucleus. They have a distinct cell outline, but no suggestion of a cell membrane. In shape they are oval to roughly polygonal according to the degree of crowding together. The cells in the sternal tumor are slightly smaller, averaging 8.5 x 5.5 microns. The nucleus is not so distinctly eccentric in position, and there is in many cells a suggestion of a cell membrane.

The cytoplasm in both is finely granular, or almost homogeneous according to variations in staining method. Some cells are finely vacuolated and vacuolation of the periphery may give the cells a ragged, frayed out appearance. The cytoplasm is weakly basophilic. In many cells can be found one or two small dots staining dark blue with phosphotungstic acid hematoxylin. These usually lie near the nucleus in a pale zone, and are interpreted as centrosomes.

The nuclei (Plate X, Fig. 9) are vesicular with a distinct outline, and average 5.7 x 5.4 microns. Chromatin is present in rather small masses scattered through the nucleus. There is a slight

tendency to peripheral grouping of the chromatin. In the central part of many nuclei there is a small rounded metachromatically staining body considered to be a nucleolus.

A few cells are larger and contain two or three nuclei. A few have smaller, shrunken, deeply staining, nuclei. Some of the latter are tinted yellow and bear a close resemblance to nucleated red blood corpuscles. However gradations between these and normal cells can be easily traced.

Mitoses are quite easily found in the sternal growth but are excessively rare or absent in sections from the orbital tumor.

Cace V (E. and E. I., 2-1177). This specimen was also given me by Dr. F. H. Verheoff with the following note from the clinical records of the Massachusetts Charitable Eye and Ear Infirmary. A male, age 50, was admitted to the Infirmary June 5, 1906. There had been a tumor above and behind the mastoid for three years. This measured 9 x 5 cm., and was firmly adherent to the skull. It was not painful and not tender. At operation it was found that the tumor was covered externally by a fibrous capsule and that it had entirely replaced the bone so that is was in contact with the brain. It was firm in consistence and its cut surface was uniform and translucent. No other tumors were found. Dr. Verhoeff obtained a faint reaction for albumose in the urine.

Microscopic Examination.—In some parts of this tumor connective tissue stroma is present in large amount (Plate XII, Fig. 14) and everywhere it is more abundant than in the other tumors. Where it is most abundant, it occurs as quite coarse bundles of wavy connective tissues fibrils, with cells crowded into the spaces between in a more or less columnar fashion. Where connective tissue is in smaller amount cells are more numerous (Plate XI, Fig. 10). In the most cellular parts stroma is present as a moderately course meshed reticulum crowded with tumor cells. Fibroglia fibrils occur in association with flattened connective tissue cells, but they are not very abundant.

The shape of the tumor cells depends largely on the amount of stroma. They may be crowded into narrow crevices between connective tissue bundles; then they are rectangular or cuboidal (Plate XI, Fig. 11). Where the spaces in which they lie are larger the cells are polygonal with one diameter greater than the other. Where very numerous, they are oval or even round. Except for these variations in shape due to pressure, the cells present a very uniform appearance.

The cell outline (Plate XI, Fig. 11) is sharp. There is no indication of a cell membrane. The cytoplasm is homogeneous, very finely granular or ground glass in appearance. Sometimes it is finely vacuolated. The cytoplasm is weakly basophilic. The average for a number of cell measurements is 9.9 x 6.4 microns. The nucleus (Plate XI, Fig. 11) is almost always eccentically placed, almost touching the cell boundary. The nuclei are round or oval, sometimes slightly irregular. Their average size is 6.9 x 5 microns. The nucleus has a very distinct outline with a slight peripheral arrangement of chromatin. Chromatin is in fairly fine masses scattered through the nucleus but is relatively not very abundant. In many nuclei a rounded central body is present which takes a reddish color with the eosin and methylene blue stain. This is regarded as a nucleolus. It has an orange red color in the aniline blue connective tissue stain, and a reddish brown tint in the phosphotungstic acid hematoxylin stain.

Near the nucleus, usually lying in a pale zone, small dots staining dark blue with the phosphotungstic acid hematoxylin stain are found. These are believed to be centrosomes.

Besides the cells described above, slightly larger cells with two or three nuclei occur. Except for size and multiplicity of nuclei they resemble the other cells. They are not numerous. There are also a few uninuclear cells larger than those first described, with a larger, more deeply staining, slightly irregular nucleus.

Typical mitotic figures are easily found in the more cellular portions, but mitosis is not very active. The tumor is moderaely vascular. There is no evidence of any association between tumor cells and connective tissue fibrils.

Case VI (M. G. H., 297) was autopsied by Dr. J. H. Wright at the Massachusetts General Hospital. The following data has been abstracted from Wright's report (*Johns Hopkins Hospital Report,* published as a Festschrift to Wm. H. Welch, 1900, 359). The patient, a man of 54, entered the Massachusetts General Hospital February 24, 1898. About a year before he had noticed a small tumor at about the level of the nipples which had grown steadily. On admission there was a rounded tumor, 11 cm. in diameter in the sternal region at about the nipple line. This tumor was rather soft and slightly pulsating. In connection with the ribs in the back were several tumor nodules. Examination with X-rays showed changes in the 5th, 6th, 7th, 8th, 11th and 12th ribs posteriorly on the left side, and in the 7th and 8th ribs on the right. Albumose was abundantly

present in the urine. During the next four months the condition of the patient remained practically stationary. The tumors of the ribs increased somewhat in size, but that in the sternum did not. In the head at the juncture of the sagittal and lambdoidal sutures a soft tumor the size of a filbert appeared. After about four months the patient began to grow rapidly weak and to suffer much pain. Finally he became unconscious and died July 26, 1898.

Autopsy showed myeloma of sternum, ribs, vertebræ, occipital and frontal bones. The gross appearance of the tumor is as follows: "A longitudinal section through the sternum shows the bony tissue, except at the extremities of the bone, almost entirely replaced by a tissue which is soft, mushy, grayish red to dark red in color, and partly moderately firm, whitish and semitranslucent." Tumor elsewhere was quite similar.

Microscopic Examination.—Sections of the tumor show stroma to be very slight in amount. There is a delicate, coarse meshed, connective tissue reticulum. Here and there are spaces with indefinite outlines filled with blood. There are also some definite blood vessels with distinct though thin walls. The tumor cells are of a surprisingly uniform appearance. They lie close together but for the most part are not crowded (Plate XI, Fig. 12). There is no great variation in size; they average 8.4 x 4.2 microns and are round or oval, frequently polygonal when in contact with each other (Plate XI, Fig. 13). The cytoplasm is almost homogeneous in the eosin and methylene blue preparations. It usually has a ground glass appearance and it is rare to find any vacuolization. The cytoplasm has a slight affinity for basic dyes though not strongly basophilic. The cells show no distinct cell membrane, though the cell outline is even and sharply demarcated.

The nucleus (Plate XI, Fig. 13) is almost always round and averages 4.2 x 4.1 microns. An eccentric position is almost constant. Chromatin masses are relatively large in size, and for the most part lie in close apposition to the cell membrane so that there is left a small central portion rather free from chromatin. Here is frequently seen a small, more refractile, rounded mass taking a pinkish stain in eosin and methylene blue preparations in contrast to the blue of the other nuclear content. This body is regarded as the nucleolus.

In sections stained with phosphotungstic acid hematoxylin many cells show in a paler zone near the nucleus one or two small, blue black dots like centrosomes.

A few cells with two nuclei occur but these are not frequent. Many cells show a shrunken deeply staining nucleus, and it is not uncommon to find cells stained with hæmoglobin. No mitoses could be found in cells in definite tumor masses, but they are found nearby in portions of bone marrow in which both tumor and other cells occur. However it is not possible to say whether these are tumor cells in mitosis or not.

Sections from all six tumors were stained in Ehrlich's triacid stain, followed by differentiation in dilute acetic acid. However in none could granules in the Ehrlich sense be brought out, though many of the cells thus stained had a more granular appearance than when stained with eosin and methylene blue. This granulation however did not suggest that of myelocytes.

The preceding cases have been described especially from the point of view of cytology. Clinical history and autopsy findings have been included only so far as was deemed necessary to justify the diagnosis of multiple myeloma. The cases are not intended to add anything to the *casuistics* of the condition. By comparing side by side the cells in these cases it is hoped that a clearer insight into the nature of the cells may be attained, and if possible the many conflicting interpretations of the nature of these cells be harmonized. It will be interesting in this connection to review briefly such cases as have been reported by others, excluding those in which cell description is given in insufficient detail. The earlier cases cannot be included owing to meagreness of histological description, and a number of more recent ones are excluded for similar reasons.

The case of Thomas (*loc. cit.*) is described here in detail from tissues obtained at autopsy. He considered the cells to be closely allied to plasma cells. Wright's case (*loc. cit.*) has been described here. Wright believes the cells to be plasma cells.

MacCallum's case (*loc. cit.*) is included in my series. He regards the cells as non-granular myelocytes.

Abrikossoff (*Virch. Archiv. f. Path. Anat.*, 1903, clxxiii, 335) describes the cells in his case as measuring 8 to 14 microns. The cells are rich in cytoplasm. The nucleus is eccentric, measures 4 to 6 microns and is round, oval or slightly irregular. Rarely the cells have two nuclei. The nuclei have a delicate chromatin network and distinct nucleolus. The photograph of an area in this case resembles Case IV of my series. Abrikossoff interprets the cells as myelocytes.

Vignard and Gallavardin (*Rev. de Chir.*, 1903, xxiii, 91) report two cases. In Case I the cells are cubical or polygonal. Cytoplasm is relatively abundant and non-granular. A drawing shows eccentric position of nucleus which is relatively small. Nuclear detail is not given. In Case II, also reported by Dubost (Thése, Paris, 1896–97) the cells are round or polyhedral. The cytoplasm is non-granular and relatively scant. The authors make no attempt to homologize the cells.

Weber (*Am. Jour. of Med. Sciences*, 1903, cxxvi, 644, and *Jour. of Path. and Bact.*, 1903, ix, 172) finds rounded or polyhedral mononuclear cells with granular cytoplasm and eccentric nucleus. Muir thinks these cells are derived from the myelocyte. MacLeod says they morphologically resemble plasma cells, but the nuclei do not show the characteristic arrangement of five or six deeply staining chromatin bodies around the periphery so generally found in plasma cells.

Ribbert (*Centralbl. f. allgem. Path.*, 1904, xv, 337) describes cells like marrow cells, some of which are tinged with hæmoglobin and resemble erythroblasts. Ribbert believes the tumor cells to be erythroblasts. His cell description is insufficient to give any adequate idea of the appearance of the cells beyond their hæmoglobin content.

Zininger (*Am. Med.*, 1904, vii, 637) finds large oval or angular cells with eccentric vesicular nuclei and a nucleolus. Sometimes two to four nuclei, separated or connected by a chromatin thread, occur. Tumor cells, according to this author, resemble the plasma cells of Unna except for larger size and vesicular nucleus.

Scheele and Herxheimer (*Zeitsch. f. klin. Med.*, 1904, liv, 57) describe round or polygonal cells with relatively large nucleus and slight cytoplasm. The nucleus is somewhat larger, more lightly staining and with more distinct chromatin net and nucleolus than in the ordinary lymphocyte. They think the cells are derived from myelocytes.

Hoffmann (*Ziegler's Beiträge z. path. Anat.*, 1904, xxv, 317) reports a case, unique in that it is a characteristic myeloma with metastasis in the liver. Extension from bone to soft parts often occurs, but with the exception of Hoffmann's case and my Case III, metastasis is not reported. The cells in Hoffmann's case are round or oval and stain intensely with Unna's polychrome methylene blue except near the nucleus where there is a paler zone. The nuclei are eccentric in position. Sometimes there are two nuclei. Hoffmann believes in the plasma cells origin of these tumor cells. His plates show cells similar to those in Case I of my series.

Sternberg (*Verhandl. d. Deutsch. path. Gesellschaft*, 1904, vi, 34, and *Zeitsch. f. Heilk.*, 1904, xxv, Path. Anat. Abth., 89) describes cells like large mononuclear leucocytes with relatively large palely staining round or oval nuclei. Chromatin is similarly arranged as in plasma cells. Sternberg demonstrates neutrophile granules in the cytoplasm and considers the cells to be myelocytes.

Aschoff (*Münch. med. Woch.*, 1906, liii, 337) in his case finds cells which in cytoplasmic staining, eccentric nucleus and coarse chromatin masses resemble plasma cells. Also there are cells similar to myeloblasts.

Jores (*Deutsch. med. Woch.*, 1906, xxxii, 863) in a brief cell description likens the cells to large mononuclear cells of the blood. The cytoplasm is non-granular and no hæmoglobin is present in the cells. He interprets them as mother cells of the leucocyte series.

Lubarsch (*Virch. Archiv. f. path. Anat.*, 1906, clxxxiv, 213) describes large round or polygonal cells with intensely stained nucleus. The cells are not granular. He considers metastasis to be possible.

Von Verebély (*Beitr. z. klin. Chir.*, 1906, xlviii, 614). Cells round or polygonal with no cell membrane but a sharp outline, occasionally ragged. The nucleus is eccentric. Cytoplasm is basophilic with clear perinuclear zone. The nucleus is round or oval with distinct nuclear membrane and mural arrangement of chromatin. Sometimes cells with two to three nuclei are found. The cells resemble plasma cells.

Menne (*Virch. Archiv. f. path. Anat.*, 1906, clxxxiii, 115) reports two cases. In Case I the cells are large and round. They have large round or oval nuclei, infrequently eccentric. Nuclei show a delicate, regular, chromatin network and distinct nucleolus. The cytoplasm is not granular. In Case II smears were made and stained, but no granules were found in the cytoplasm. No hæmoglobin was in these cells. Menne believes the cells are pseudomyelocytes.

Verhoeff (*loc. cit.*) interprets the cells in his case, one of those studied here, as plasma cells. All these tumors were very cellular and stroma was scant.

These cases in so far as may be judged from their descriptions show a quite uniform cell type, though there is considerable minor variations in cells in the different tumors. For eight of the cases it is claimed by their reporters that the cells find a close analogy in the plasma cell. Of six it is believed that the cells bear a close relation to immature myelocytes. In one, the case of Sternberg, cytoplasmic granulation is described. In Ribbert's case an erythroblastic nature is claimed. In some cases no attempt to homologize the cells was made. The question arises, How far in light of these cases can the histogenetic source of the tumor cells be determined?

Myelomata develop in the bone marrow, presumably from some cell or cell group normally present within the bone marrow, if they follow the laws which are believed to govern tumor formation in general. If this is the case, then in the bone marrow should be found cells of analogous appearance. Identity of cell structure is not to be expected, for the wide range of possible variation in tumor cells from their ancestral cell type is well known. The problem of determining the cell relations of the myeloma cell is all the more difficult because in bone marrow there is such a variety of cell forms.

A study of the bone marrow from a variety of cases shows that but two cell types have any very close resemblance to the cells of these myelomata. Both are cells which have a slightly basophilic cytoplasm, distinct cell outline but no cell membrane. Both have

a nucleus, frequently eccentric in position, with a distinct nuclear membrane and fairly constant nucleolus. In the one the nucleus is pale staining with fine chromatin masses generally scattered throughout the nucleus, sometimes clumped more or less toward the center, but without any tendency to a mural arrangement. These cells (Plate XII, Fig. 17) belong to the myelocyte series, an early stage of development, usually before the specific granulation in the Ehrlich sense takes place, and are numerous in all active marrows. They may be called premyelocytes.

In the other cell (Plate XII, Fig. 15) the nucleus stains more deeply, the chromatin masses are coarser, there is rarely any central clumping of the chromatin but a strong tendency to a mural arrangement of chromatin masses. A nucleolus is a more constant feature. These cells appear to be constantly found in bone marrow. They are usually infrequent and never in large numbers. The maximum that I have ever found are scattered groups (Plate XII, Fig. 16, two cells in focus) of five or six cells with a few single cells so that about every other field of a Zeiss 2 mm. objective and 4 ocular would show at least one of these cells. This was in cirrhosis of liver (1 case), staphylococcus pyemia (1), pernicious anemia (1), pyonephrosis (1), organizing pneumonia and glomerulo-nephritis (1), carcinoma of prostate (1). I have found them fewer in number, generally singly, in cases of pyelonephritis (1), myelogenous leukemia (1), tertiary syphilis and acute bronchopneumonia (1), arteriosclerosis and heart hypertrophy (1), acute bronchopneumonia (1). I have never been able to find any evidence of developmental changes in these cells. Their nature is not certain. They seem to have no relation to the formation of erythrocytes or polynuclear leucocytes. Of their relation to the lymphocyte series I can give no positive evidence. They appear closely related. Their closest analogue outside of the bone marrow is the plasma cell from which they differ in being slightly larger, both cell body and nucleus, in having a less homogeneous ground glass cytoplasm, in being rather less basophilic, in having not quite so coarse chromatin granules, but all these differences are of minor degree and do not serve to distinguish sharply these cells from the plasma cells of inflammatory tissue. However as there

are these slight differences, I prefer to speak of these cells in the bone marrow as bone marrow plasma cells, and regard them as in some way related to the lymphocyte series of cells.

Which of these cells stands closest to the myeloma cell? As is evident from the description of the cells in the myelomata reported by me and by others, there is considerable variation in the cell picture. The features most constant in the tumor cells are cytoplasm finely granular or slightly vacuolated, slightly basophilic; a nucleus eccentric in position, with a tendency to a mural arrangement of chromatin and a nucleolus; the presence of centrosomes. There is some variation in cell size, the average of measurements in each case being:[7]

Case VI	cell body	8.4 x 4.2	microns;	nucleus	4.2 x 4.1	microns.
Case IV sternal part	"	8.5 x 5.5	"	"	5.7 x 5.4	"
Case IV orbital part	"	9.4 x 7.6	"	"		"
Case V	"	9.9 x 6.4	"	"	6.9 x 5	"
Case II	"	10.4 x 6.8	"	"	6 x 6	"
Case III	"	10.5 x 6.8	"	"	7.2 x 6.4	"
Case I	"	11.4 x 7.7	"	"	8.1 x 7.2	"

To compare with these I have made measurements of a number of cells in the bone marrow. The average of these is:

For myelocytes and premyelocytes:						
Case of pernicious anemia	cell body	11.5x9.25	microns;	nucleus	8 x7.5	microns
Case of acute bronchopneumonia	"	11.8x8.25	"	"	7.2x6.5	"
Case of cirrhosis of liver	"	12.25x9.7	"	"	8.5x7.2	"
For bone marrow plasma cells:						
Case of pernicious anemia	cell body	9.7x7	"	"	5.1x4.6	"
Case of acute bronchopneumonia	"	8.6x6.6	"	"	5.3x5	"
Case of cirrhosis of liver	"	9.9x7.5	"	"	5.6x4.8	"

From these measurements it is evident that cell body and nucleus of the myelocytes are rather larger than those of the tumor cells. The bone marrow plasma cells agree in size with the cells of those tumors which have the smaller sized cells (Cases VI, IV and V). A glance at the cell descriptions in these will show that these tumors have cells which agree closely in structure with the bone marrow plasma cells. (Compare Fig. 15 with Figs. 9, 11 and 13.) The other tumors have cells which while retaining the characteristics

[7] All these measurements were made on cells in tissue hardened in Zenker's fluid, embedded in paraffin and stained with eosin and methylene blue. The same lenses and micrometer were used throughout.

in part of the plasma cells yet show differences. Still these differences though in the direction of the premyelocyte do not give a cell so large as the premyelocyte, and when compared side by side the cells appear to be more closely homologous with the bone marrow plasma cells than with the premyelocytes. This is certainly true also for the cells in many of the tumors reported by others.

In other words it seems possible to arrange the cells of these tumors in series so that between the cells of succeeding cases the differences are slight, though the difference between the cells at the extremes are fairly great. (As such a series take the cells in the following order, Fig. 3, 7, 11, 5, 9 and 13, ending with Fig. 15, a bone marrow cell.) The cells of one tumor (Case VI) described by me are almost identical in structure with plasma cells of the marrow, and those in two others (Cases IV and V) are but very slightly different. The cells in the other cases seem to me to present more similarity to the plasma cell than to the myelocyte. If the myelocyte is assumed to be the ancestral cell the departure from the type is very much greater. (Compare the premyelocyte in Fig. 17 with cells in the series given above.) It is probably not possible to give any convincing proof of the ancestry of these cells. The cases studied side by side and in comparison with bone marrow cells leave the impression that we have in these tumors a development from certain bone marrow cells, which I have considered to be bone marrow plasma cells. Whether this is true for all reported cases is exceedingly difficult to say, especially for those cases in which the cells are not described in much detail. Possibly some cases, as that of Sternberg in which neutrophilic granules are described, may have to be regarded as an exception.

Ribbert's (*loc. cit.*) contention that the case studied by him was composed of erythroblastic cells is not supported by these cases. The frequent presence in my cases of cells stained with hæmoglobin and resembling nucleated red blood corpuscles as already described suggests the possibility that a similar condition may have been present in Ribbert's case. In my cases it seems very certain that these cells result from a combination of cell degeneration and hæmoglobin staining. Such hæmoglobin staining of plasma cells

is by no means uncommon, especially in the spleen where I have observed it many times, and this fact may be taken as some slight further evidence of the plasma cell nature of the tumor cells.

There is nothing in these tumors to suggest any transformation of cells along the line of development from premyelocyte to polymorphonuclear leucocyte. Sternberg is the only observer who has succeeded in demonstrating neutrophile granules in the cells of a myeloma. This fact in this single case does not seem sufficient evidence on which to base the opinion that all myeloma cells are myelocytes in some stage of development when we consider the great similarity of the cells in very many cases to plasma cells. If more cases like Sternberg's are accumulated, it would justify a subdivision of myelomata into (*a*) those with cells resembling plasma cells, and (*b*) those with cells resembling myelocytes. Even then it would still be possible without claiming a histogenetic relation between plasma cell and myelocyte to form a tumor group, having the main characteristics already described for the myelomata, in which all cases could be arranged in series with a tumor composed of cells identical with plasma cells at one end of the series, and a tumor with cells identical with myelocytes at the other, while between would come cases whose cells represented a gradual transition, from one to the other. Such a group would contain tumors showing no greater differences than occur in all other tumor groups such as sarcomata, carcinomata, etc. However with regard to the tumors actually studied by me all would be grouped at the plasma cell end of such a series.

SUMMARY.

A histological study of six cases of myeloma shows a striking similarity of cell structure with differences of minor degree. The common characteristics of the cells are a finely granular, slightly basophilic cytoplasm, an eccentrically placed nucleus, a nucleolus, centrosomes, a nuclear membrane and a tendency to mural arrangement of the chromatin. With these characteristics there is a greater resemblance of the tumor cells to bone marrow plasma cells than to myelocytes. In most of the cases reported by other observers the cells show these characteristics and these tumors form a group within which the cells of individual cases show no greater variation than occurs in other tumor groups.

Fig. 1.

Fig. 3.

Fig. 2.

Fig. 5.

Fig. 4.

FIG. 7.

FIG. 6.

FIG. 9.

FIG. 8.

FIG. 11

FIG. 10.

FIG. 13.

FIG. 12.

FIG. 15.

FIG. 14.

Plasma cell

Eosinc
myelo

emyelocyte

FIG. 16.

FIG. 17.

DESCRIPTION OF PLATES IX–XII.

FIG. 1.[8] Rib from Case I.

FIGS. 2, 4, 6, 8, 10, and 12. Low power (425 diameters) fields from the six tumors to show cellular character and scant stroma.

FIG. 14. (425 diameters.) Fibrous portion of tumor from Case V.

FIGS. 3, 5, 7, 9, 11, and 13. Cells (1500 diameters) from each tumor.

FIG. 15. Bone marrow plasma cell from a case of pernicious anæmia. (1500 diameters.)

FIG. 16. Group of bone marrow plasma cells from a case of cirrhosis of the liver. (1500 diameters.)

FIG. 17. Bone marrow from case of cirrhosis of liver. Shows premyelocyte, plasma cell and eosinophilic myelocyte. (1500 diameters.)

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[8] For all these photographs I am indebted to Mr. L. S. Brown of the Massachusetts General Hospital.

THE HISTOLOGICAL LESIONS OF EXPERIMENTAL GLANDERS.[1]

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Research into the histological lesions of experimental glanders has hitherto failed to yield comprehensive results. The literature of the subject is scanty and affords at best an inadequate description of the acute lesions. The views held by various observers regarding the histology of experimental glanders are conflicting and observations upon the tubercle of this infection are extremely contradictory.

Baumgarten[2] states that the histogenesis of the tubercle of experimental glanders is essentially the same as that of the miliary tubercle differing only in that the former undergoes necrosis more quickly than the latter. He holds that the primary effect of the glanders bacillus upon a tissue is the proliferation of the "fixed cells" with secondary degenerative change and not destruction of tissue and polynuclear invasion. Wright,[3] on the contrary, concludes that the experimental glanders tubercle and the miliary tubercle are not analogous in their histogenesis, and states that there is primary necrosis of the tissue followed by exudation, which is frequently of a suppurative character. Tedeschi,[4] probably working with cultures, in virulence intermediate between those used by Baumgarten and by Wright, concluded that the primary effect of the bacillus of glanders was necrosis of the tissue with leucocytic invasion.

These conflicting observations have led us to undertake the following experimental work in the hope of determining whether or not the histological changes in experimental glanders vary directly with the virulence of the organism employed.

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[1] Read before the Association of American Physicians, May 8, 1907.

[2] Lehrbuch der path. Mykologie, 1885.

[3] *Jour. of Exper. Med.*, 1896, i, 577.

[4] *Zeigler's Beiträge*, 1893, xiii, 365.

METHODS.

The animals employed throughout this work were rabbits and guinea-pigs. The methods of inoculation were subcutaneous, intravenous, intraperitoneal, intra-orbital and corneal. The inoculations are grouped in series according to the mode of inoculation and the virulence of the culture employed.

Rabbits were the only animals employed for corneal inoculations. Two methods were used, (1) inoculation with parallel incisions, and (2) subepithelial injection. After etherization the eyeball was steadied with fixation forceps, care being taken not to injure the cornea. Under aseptic precautions three parallel incisions into the corneal substance were made about 2 mm. apart and well away from the sclero-corneal junction. The cornea thus prepared was then inoculated with the glanders culture by means of a platinum needle.

With the second method a suspension of bacilli was injected beneath the corneal epithelium by means of a sterile hyperdermic needle at a distance from the point of entrance. With both methods one eye was used as a control in every instance. The animals were afterwards killed at intervals varying from fifteen minutes to three weeks.

The same strain of bacillus Mallei was used throughout the work which extended over a period of two years. The animals were injected with varying amounts of a highly virulent culture, of a moderately virulent culture, of an attenuated culture, and of a culture killed by heat.

The attenuated culture was obtained by frequent transplantation during a period of eighteen months. It is interesting to note that the bacilli of lowered toxicity change remarkably in their morphological and cultural characteristics. They lose their short plump appearance and become thin, elongated rods with definite clear spaces at regular intervals along the body. On the ordinary culture media such as potato, agar-agar, blood serum, etc., the culture within twenty-four hours becomes extremely dry, tenacious and stringy; it is so tenacious that it is difficult to take off a part of the growth with a platinum loop, the whole surface growth coming away *en masse*. In our experience these peculiarities of the surface culture are characteristic of the period of non-virulence.

The highly virulent bacilli were obtained by frequent passage through a series of guinea-pigs. Devitalized bacilli were prepared by suspending in fifteen cubic centimeters of physiological saline solution a twenty-four hour growth of a virulent agar slant culture and heating the suspension for two hours at 56° C.

The bacilli were prepared for inoculation from a twenty-four hour growth upon one per cent. dextrose agar slant by washing with fifteen cubic centimeters of bouillon or salt solution and thoroughly shaking to ensure an even emulsion. The dosage varied with the virulence of the bacillus and the animal employed, whether rabbit or guinea-pig.

In general the dosage was as follows:

1. Of attenuated culture, 3 c.c. for guinea-pigs and 10 c.c. for rabbits.

2. Of moderately virulent culture, 1 c.c. for guinea-pigs and 5 c.c. for rabbits.

3. Of highly virulent culture 0.5 c.c. for guinea-pigs and 2 c.c. for rabbits.

For the corneal injection a very much smaller amount was employed. Here the exact quantity of unsuspended bacilli was not accurately determined, but approximately it amounted to an injection of 0.1 cubic centimeter in each case.

In animals partially immunized to the virulent glanders bacilli larger doses were finally employed. This partial immunization was effected by administering every three or four days during a period of four weeks, gradually increasing doses of the virulent organism.

As stated by one of us in an earlier publication, it is only occasionally that rabbits succumb to experimental inoculation with living virulent glanders bacilli and they are far less susceptible to this form of infection than are guinea-pigs. The rabbit stands large and repeated doses. Pregnant rabbits and mothers are much more susceptible. Though guinea-pigs are very prone to glanders infection it is noticeable how much less susceptible is the female than the male.

For histological study the tissues were fixed in Zenker's fluid, alcohol, or formalin and embedded in paraffin. Eosin-methylene-

blue was used in the routine staining of sections. Mallory's phosphotungstic-hæmotoxylin and connective tissue stains were employed for the special differentiation of tissues. Sharlach R. was employed to demonstrate the presence of fat. As a routine stain the eosin-methylene-blue (Mallory) was by far the most satisfactory.

The staining of the bacteria in the tissues was accomplished by means of a special method which is described elsewhere in the present number of this Journal.

HISTOLOGY.

In the following histological description of the lesions in experimental glanders the organs will be considered separately.

Cornea.—Owing to the absence of blood vessels and the peculiar histological structure of the cornea this organ was selected as being the most suitable for the study of the earliest tissue changes.

The cornea of the rabbit was used in all three sets of experiments owing to the greater resistance of this animal to glanders infection compared with the guinea-pig and each experiment was repeated in order to control the results.

In the first series, in which the highly virulent culture was used, the animals were killed at varying periods ranging from half an hour to forty-eight hours after inoculation. In the second series, in which the culture employed was of moderate degree of virulence the rabbits were killed at varying intervals extending over a period of five days. In the third series, inoculated with the attenuated culture, the animals were killed at intervals extending over a period of one month.

The lesions thus produced in the cornea fall readily into two distinct groups, viz., the acute exudative and the chronic proliferative, the determining factor being the virulence of the culture.

The corneal lesion resulting from the introduction of the highly virulent bacillus is to be described as inflammatory with marked disintegration of the tissues and of the invading leucocytes. The disintegration was often observed about the site of injury within half an hour of inoculation and before the migrating leucocytes from the vessels of the limbus had time to approach—a destruction so severe in some cases as to render unrecognizable the individual cells comprising the lesion.

The character of the acute inflammatory lesion varies to some extent with the time elapsing between the inoculation and the killing of the animal. The

vessels in the "danger zone" of rabbits killed in fifteen minutes after the corneal inoculation show no evidence of change. There is no apparent dilatation of either the vessels or the perivascular spaces and there is an absence of inflammatory cells migrating towards the site of injury. Nevertheless at the site of inoculation there is a marked disintegration of the corneal tissue into cell fragments and deep blue homogeneous chromatin-like masses of varying size, apparently the result of a direct action of the glanders toxin upon the tissues. These chromatin bodies have been described by various investigators as characteristic of the glanders nodule. The absence of polynuclear cells approaching the site of injury, and the fact that the vessels in the sclero-corneal zone are as yet not dilated together prove that the detritus and chromatin bodies are the product of nuclear elements derived from the disintegrated cells of the injured area. After a few hours the injured area becomes invaded with the inflammatory cells from the vessels of the limbus, which in turn suffer fragmentation and disintegration.

The cornea inoculated with the bacilli of moderate virulence affords an excellent opportunity to study the nature and origin of the cells comprising the acute exudative lesion. In this series of experiments the toxin was not of sufficient power primarily to destroy the tissue or the invading leucocytes.

By means of a special method we were able to determine the number and situation of the glanders bacilli and their relation to the various cells. The fact that the bacilli remain local with no tendency to spread through the tissues explains the focal character of the lesion.

The most interesting feature of the early lesion is the appearance of peculiar acidophilic cells, which collect in considerable numbers about the site of inoculation as early as fifteen minutes after the introduction of the bacilli. At this period the incised corneal epithelium is gaping and the corneal spaces contain numbers of cells with large deep-staining red granules. There is no evidence that these cells have come from the blood vessels. They are interesting in view of their nature and origin, and of the possible rôle played in inflammations produced by certain toxins and chemical agents. These leucocytes are generally polymorphonuclear though sometimes the nucleus is similar to that of the transitional leucocytes.

The nucleus is surrounded by twenty or thirty closely arranged coarse granules which stain intensely with eosin. These cells differ from the polymorphonuclear leucocytes of the rabbit, (1) by the size and staining reaction of their granules, (2) by their appearance in the corneal lesion long before the vessels in the limbus (from which the ordinary pus cell migrates) react, and (3) by the fact that they are not phagocytic for the invading bacilli.

These large acidophilic granule cells apparently come from the tissues in the neighborhood of the injury or enter through the corneal incision from the conjunctival sac. For in the earliest lesion there is an absence of any change in the nearest vessels and a wide clear field intervenes between the vessels and the outermost cells about the lesion. Following the early injury to the cornea these cells are the first to respond to the bacterial invasion and

practically constitute the histological lesion. (See Plate XIII, Fig. 2.)

Opie[5] states that the polynuclear eosinophile cell rarely, if ever, ingests bacteria. Kanthack and Hardy,[6] however, claim for the eosinophile cells a primary attack directed against the invading bacteria. There is every reason to believe that the large granule cells attracted early to the corneal injury are eosinophilic in nature; and their failure to take up bacteria would indicate the absence of defense against bacterial invasion.

The polynuclear leucocyte of the rabbit whose granules are smaller and less deeply stained can be traced from the vessels in the sclero-corneal junction in an unbroken path along the corneal spaces just beneath the epithelium, to the site of the injury where they become actively phagocytic for the bacteria.

The first vascular change noted in the sclero-corneal angle occurs about one hour after the inoculation of the cornea, and consists of a dilatation of the vessels and of the perivascular spaces, with an accumulation of polynuclear cells within the lumen and their migration into the surrounding tissues. Simultaneously with the migration of the leucocytes about the vessel collect numerous lymphoid and plasma cells, which apparently do not follow the polynuclear cells to the site of the injury but remain in the tissue about the vessel.

The control eye in each case shows no reaction about the incision for six hours or more. Where the incisions penetrate only a short distance into the stroma the repair is complete in less than twelve hours, as indicated by the congealed epithelial surfaces without the least sign of an inflammatory infiltration. Often the injury is recognized only by the mass of heaped up epithelial cells projecting below the limit of the normal corneal epithelium.

Following inoculation of the bacilli of low degree of virulence the proliferative type of lesion ensues. The best results were obtained by deep corneal injection of the bacilli by means of a fine needle without serious injury to the corneal epithelium. (See Plate XVI, Fig. 14.) The earliest change noted is swelling of the

[5] *Johns Hopkins Hospital Bulletin*, 1904, xv, 71.
[6] *Jour. of Physiol.*, 1894–1895, xvii, 81.

corneal corpuscles and accumulation of moderate numbers of polynuclear eosinophiles. Later the epithelioid cells collect and give rise to a more or less well defined focus. The microscopic picture in from five to seven days after inoculation is similar to that of the lesion produced by the tubercle bacillus. The lesion consists of numerous cells of an epithelioid nature and moderate numbers of lymphoid and polynuclear cells. Giant cells were not observed in the lesions of the cornea. The occurrence in the proliferative type of corneal lesion of epithelioid cells and the absence of giant cells, whereas in the other organs under similar conditions the two types of cells are associated, would indicate a connective tissue histogenesis for the epithelioid cell. On the other hand the lesions in the region of the limbus contain multinucleated cells associated with epithelioid cells.

Liver.—The lesions in this organ in rabbits and guinea-pigs differ in no essential feature. The extent and character of the lesion depend upon the virulence of the culture. In a large number of animals the liver shows well-defined changes, presenting microscopically the picture of miliary tuberculosis. In general the gross changes consist of discrete, glistening, grayish-white foci varying in size from minute points to areas one millimeter in diameter. These areas are invariably found in greater numbers just beneath the capsule of the liver but in no case where the areas are seen on the liver surface are they absent from the deeper parts of the organ. In a number of animals however no macroscopic changes were discernible although subsequent microscopic study revealed definite lesions.

Microscopically sections of liver tissue show a gradation in the lesions varying with the culture employed, the mode of inoculation and the susceptibility of the animal, the virulence of the culture playing the most important rôle. In general these lesions are of three types: (1) the acute inflammatory, (2) the degenerative, and (3) the proliferative.

Inoculation of the ear vein of a rabbit with the highly virulent culture produces acute miliary abscesses of the liver. The partially attenuated culture similarly injected at repeated intervals gives rise to focal lesions of a proliferative character. The acute inflammatory lesions in the liver consist of foci in which the cells have been destroyed and subsequently invaded by polymorphonuclear leucocytes. These leucocytes are often so closely packed that the fixed cells of the part are made out with difficulty. The lesion varies in structural character from a focus composed of fragmented and disintegrated nuclei and protoplasmic masses, to one in which the cell collection is well preserved.

The lesions of intermediate grades are met with in animals which have received repeated inoculations. In general it may be stated that the difference in severity noted in the acute inflammatory lesions depends upon the toxicity of the culture. The miliary abscesses are always sharply defined and do not differ (except possibly in the sharpness of definition) from acute miliary abscesses in this organ resulting from other infecting agents.

The more severe type of lesion presents the picture described by most observers as characteristic of the experimental lesion. The microscopic appearance consists of a collection of large and small deeply staining chromatin masses, scattered nuclei and protoplasmic fragments. Fibrin is present in variable amount. The glanders bacilli are readily distinguished by a special staining method as small rods arranged singly and in pairs in the center of the focus. Although the liver cells within the lesion cannot be distinguished, those at the periphery remain unchanged, that is, the necrotic zone is sharply defined by apparently normal cells.

The less severe form of the acute lesion is composed of polynuclear leucocytes in a good state of preservation. In this type of lesion parts of liver cells and occasionally whole liver cells, though necrotic, are readily distinguished. There is an absence of "chromatic globules" and of the macerated appearance so characteristic of the tissue changes in the more severe type of lesion. Occasionally an isolated necrotic liver cell within the focus contains several bacilli and threads of fibrin.

The miliary abscesses of the liver are found in no particular site; they occur either intralobular or interlobular. However the lesion within the lobule is smaller and more sharply defined. There is less tendency for the intralobular foci to spread and coalesce with neighboring foci. Many of the lesions are extremely small, consisting often of less than a dozen closely collected polynuclear cells. These early intralobular collections of leucocytes seem to form within the subsinusoidal space. More commonly the lesion begins as a collection of leucocytes within the capillary space, being preceded by an injury to the lining endothelium, indicated by the occurrence of fibrin about the swollen, deeply stained lining cells. In some of the early lesions the leucocytes are collected about large mitotic endothelial cells still attached to the capillary wall.

In the sections of liver tissue the bacilli occur in association with the lesion. In the intravenous series of animals killed forty-eight hours or more after inoculation there is a tendency for the bacilli to lodge along the vessel wall. From a careful study of the early acute lesions it is obvious that the bacilli lodging along the route of the portal circulation either by direct action or the action of their toxin injure or destroy (depending on the virulence) the lining endothelial cells and cause on the one hand a focal leucocytic collection, or on the other a proliferation of the endothelium. This proliferation of the endothelial cells of the capillaries plays an important rôle in the formation of the glanders tubercle, especially in connection with the origin of the giant cell.

The degenerative lesions of the liver will be considered under two headings: (1) focal necrosis, and (2) vascular changes.

Focal necrosis of the liver occurs regularly in the rabbit and guinea-pig which have been inoculated with the moderately virulent bacilli. The central and zonal types of necrosis rarely occur. The most extensive lesion develops

in the animals which have been partially immunized by repeated intravenous injections. As a rule the liver necrosis does not develop earlier than seven to ten days after the first inoculation.

In a rabbit that died of glanders infection fourteen days after the first inoculation all the lobules are affected, and in many the necrosis comprises more than half the lobule. (See Plate XVI, Fig. 15.) The occurrence of focal necrosis apparently depends upon the degree of virulence of the culture. The lesion never occurs in animals infected with the highly virulent culture except in those partially immunized and subsequently receiving a fatal dose. In guinea-pigs the lesion is less common than in rabbits and usually occurs after the repeated injection of the attenuated culture over a period of weeks.

The areas of necrosis vary from a single cell to areas composed of many cells. There is apparently no selection of special parts of the lobule. The same section may show focal areas of necrosis in the central, mid-zonal and peripheral parts of the same lobule. Not infrequently a single necrotic cell occurs between normal liver cells with which it is sharply contrasted as it stains more intensely with eosin. In some areas of necrosis the liver cells contain large and small vacuoles; and the failure of these to stain with Sharlach R, would indicate "vacuolar" and not fatty degeneration. Some of the vacuoles contain fibrin threads either single or arranged in crossed bundles. Occasionally the vacuoles contain an eosinophile cell or a polymorphonuclear leucocyte. The absence of an inflammatory reaction in connection with the focal areas of necrosis is remarkable; even in the older lesions where the liver cells have lost their nuclei and appear as deeply stained eosin masses which are only distinguished as liver cells by the shape and size, there is rarely any polynuclear invasion.

In addition to this form of focal necrosis, the result of the action of the strong toxin, there is another form of necrosis which undoubtedly results from an inflammatory exudate collecting about an area of liver cells. In a guinea-pig which received intraperitoneally a highly virulent culture and succumbed to the infection on the fourth day, the liver presented focal areas of necrotic cells surrounded by well defined zones of polymorphonuclear leucocytes. These areas contained twelve to thirty swollen, deeply stained liver cells, many of which were without nuclei, and the whole mass was encircled by a broad zone of acute inflammatory exudate. The central mass of degenerated liver cells remained remarkably free from leucocytes.

The eosin-methylene-blue stain is admirably suited for the demonstration of the focal lesions in the liver. The earliest change in the liver is indicated by the tendency of the cells to stain more intensely with eosin. At first the nuclei stain a deep purple blue in contrast to the light blue of the normal nucleus; then they stain less intensely until finally the nucleus is lost. Many of the liver cells though completely degenerated preserve their shape and size long after all trace of the nucleus has disappeared.

The protoplasm of the liver cells, the seat of necrosis, is often converted into curious products of degeneration. These products occur in three distinct variations. In one form the protoplasm of the liver cells undergoes a curious transformation into numbers of well defined and separated "chromatoid bodies" resembling in their structure the nucleus of a lymphoid cell; they differ, however, in that they are smaller and stain intensely with eosin. There is also a

resemblance to blood platelets though the latter are more angular in outline, whereas these protoplasmic bodies are spherical. These curious structures may number twenty or more in a cell, and are often arranged to form perfect geometrical figures. They occur only in the liver cells with the more advanced stage of necrosis.

The second group of protoplasmic bodies in necrotic liver cells appear as small and large globules (the smaller undoubtedly coalesce to form the larger). These globules stain intensely with eosin and are homogeneous throughout. This form of cell degeneration is more frequently met with than the form above described.

The third variety of degeneration product appear as finely granular masses filling the necrotic liver cell. This granular conversion of the protoplasm begins soon after the cell loses its nucleus and presents the earliest change in the cell protoplasm.

It would seem that focal necrosis of the liver in experimental glanders results chiefly from a plugging of the interlobular capillaries with fibrinous thrombi. In the vessels adjoining the necrotic areas of liver cells masses of fibrin either partially or completely occluding the lumen can be demonstrated.

Occasionally large phagocytic cells and giant cells are found in the capillaries connected with the focal areas of liver necrosis. Here the sinusoids are narrowed owing to the swelling of the parenchymal cells and the accumulation of serum in the subsinusoidal spaces.

Masses of fibrin also occur in the capillaries of these necrotic areas. Frequently the lining endothelial cells contain mytotic figures which would indicate some special action of the toxin on the vessel wall. The dividing cells later become detached and escape into the lumen where they assume a phagocytic rôle. It is reasonable to suppose that the greater number of phagocytic cells found free in the blood channels of the liver originate in the endothelium of the liver.

The bile radicles in the areas of necrosis are often dilated and show aneurysmal bulgings.

Vascular changes occur in the perivascular vessels and in the central veins of the lobules. Here the muscle fibres are often in an advanced stage of degeneration. However in some vessels only small groups of muscle fibres or single isolated fibres show alteration. The fibres in the earlier stages of degeneration are filled with fine particles which take the Sharlach R. stain for fat. The

vascular lesions are well marked in the lobules where the parenchymal cells are apparently normal.

The proliferative changes in the liver occur in animals inoculated with the attenuated culture and in animals receiving repeated inoculations. The transition from the acute inflammatory type of lesion to the chronic proliferative lesion is striking, and is the result of alteration in virulence of the culture. The liver offers an excellent opportunity to follow the various changes in the character of the lesion from the acute focal destructive type to the chronic focal proliferative type. Though lesions of these two types differ essentially in their cells they are alike in that they are both focal in character. The lesion produced with the highly virulent culture simulates an acute miliary abscess, while the lesion produced by the attenuated culture resembles the miliary tubercle.

The glanders tubercles in the liver occur as discrete miliary foci composed of epithelioid and lymphoid cells associated with giant cells. Just as the lesions of acute miliary tuberculosis vary in character for the same case, so also varies the glanders tubercle. Out of a series of twenty-four rabbits eighteen showed proliferative lesions of the miliary tubercle type. The animals were inoculated intravenously or intraperitoneally and killed twelve to twenty-four days after the first inoculation. In fourteen cases the lesions of the liver were visible to the naked eye as discrete grayish-white tubercles studded throughout the organ, but most marked just beneath the capsule. On section they were translucent and of firm consistence. Microscopically they are focal areas composed chiefly of epithelioid cells arranged more or less concentrically. Giant cells occur in the lesions as a rule, about the periphery of the tubercle. In some areas three or more of these multinucleated cells were present, and in one tubercle there were eight. These giant cells often contain twenty or more nuclei which usually occur in the center of the cell. The same section not infrequently would contain lesions of different ages, the older foci showing necrosis. This necrosis of the glanders focus in no way resembles the necrosis of the acute inflammatory lesion in which fragmentation of nuclei and chromatin globules are so characteristic, but resembles the necrosis of the tuberculous lesions.

Lungs.—The lungs are not as frequently a site of pathological change as the other organs of the rabbit and guinea-pig. However when lesions occur they are significant, especially the proliferative type, owing to analogy with the lesion of tuberculosis. Pulmonary lesions occur in the guinea-pig more frequently than in the rabbit. Here as in other organs the type of lesions depends upon the virulence of the culture and the susceptibility of the animal, though the former is by far the more important determinating factor. The manner of inoculation does not materially influence the character or frequency of occurrence of the pulmonary lesion.

The lesions arising in the lungs are exudative and proliferative. The proliferative lesions are in the form of miliary tubercles and are best developed in guinea-pigs killed two to three weeks after the first inoculation. In three cases the pigs had received more than two inoculations. The animals were partially immunized by injecting small doses of the attenuated bacilli and then given the fatal dose of a virulent culture.

The lesions of an exudative nature occur in the form of miliary abscesses scattered throughout the parenchyma and result from the injection of the highly virulent bacilli. The extent of destruction in the pulmonary tissues, as in other organs, depends upon the virulence of the bacilli. In general, bacilli of a low grade of virulence give rise to focal proliferation; bacilli of a moderate degree of virulence produce focal lesions of an acute exudative character in which the cells are well preserved; the bacilli of exalted virulence cause disintegration of the tissues, which occurs as areas filled with fragmented nuclei and degenerated protoplasm.

Often there is no appreciable change in the gross appearance of the lungs which on microscopic examination contain definite focal lesions.

The protocols are here given of animals which well represent the two types of lesions.

Guinea-pig VII.—Female, weight 550 grams, was inoculated intraperitoneally on October 18 with 0.5 c.c. of a twenty-four hour culture of attenuated virulence suspended in 2 c.c. of bouillon. Slight area of induration appeared about the site of inoculation at the end of the second week; animal was in good health. Second inoculation was given into the peritoneum October 30; the animal was killed November 2; the weight being 440 grams. Autopsy shows an area of

induration of the skin and subcutaneous tissues about the site of inoculation. A few Gram negative bacilli are found in the smears. The tunica vaginalis is swollen and œdematous and on section thickened and studded with discrete and conglomerate pin-head and smaller nodules. The omentum is curled upon itself, and on separating the folds the serous surface contains innumerable discrete, glistening grayish-white tubercles 1 mm. to 2 mm. in diameter. Beneath the capsule of the liver and spleen are similar grayish-white nodules; many of these areas are too small to be detected in the deeper parts of the organs. The peritoneum is studded with tubercles, though they are relatively few in number as compared with those in the omentum. The inguinal and mesenteric glands are enlarged. The heart, kidney and adrenals show no visible change. The lungs are voluminous and crepitant throughout, though the whole subpleural surface presents scattered, glistening grayish-white tubercles 1 mm. in diameter. These tubercles, though present in the deeper parts of the lungs, are not as readily distinguished as those under the pleura. The mediastinal nodes are enlarged and on section exhibit areas of necrosis. A pure culture of *B. mallei* was recovered from the heart's blood.

Guinea-pig XI.—Weight, 555 grams. Inoculated subcutaneously October 30 with 0.5 c.c. of a 1 c.c. bouillon suspension to which one loop of a twenty-four hour culture of moderate virulence had been added. The animal died November 9 of general septicæmia. The autopsy showed a large caseating area at the site of inoculation. The glands were not enlarged. The peritoneal cavity presented diffuse fibrinous peritonitis. The liver contained three small pin-head sized nodules. The spleen was somewhat enlarged and congested. The heart and kidneys were normal in appearance. The testes and tunic were studded with glanders nodules. Both lungs, though voluminous, dry and crepitant throughout, were thickly seeded with innumerable discrete grayish-white translucent tubercles 1 mm. in diameter. These areas were apparently more numerous just beneath the pleura, and gave to the surface a rough finely granular appearance. The adrenals showed similar lesions beneath the capsule and in the substance of the glands.

Guinea-pig VI.—Weight 555 grams. Inoculated subcutaneously on October 3 with 0.5 c.c. of a twenty-four hour culture of low degree of virulence. Two weeks after the first inoculation the animal was in good health. The second inoculation was given subcutaneously October 18 with double the amount of the first dose. The animal weighed 585 grams and was in good health one week after the second inoculation. A third dose was given intraperitoneally on October 25. The amount of the dose was 1 c.c. of a moderately virulent culture suspended in 5 c.c. of bouillon. Five days after the inoculation the animal developed the testicular lesion, and was killed seven days after its appearance.

The autopsy revealed a small area of induration at the recent site of inoculation. The inguinal and axillary glands were enlarged. The omentum contained a few discrete grayish-white tubercles; otherwise the peritoneal cavity showed nothing remarkable. The mesenteric and bronchial lymph nodes were slightly enlarged. Beneath the capsule of the spleen and liver there were numbers of discrete grayish-white tubercles 1 mm. in diameter. The adrenals and kidneys were negative. The testes and tunica vaginalis presented extensive induration and caseation. The lungs were voluminous and crepitant except for a slight amount of œdema in the dependent portions.

The focal distribution of the lung lesions is especially characteristic. It will be noted that they are focal in each animal and occur in the form of discrete tubercles. In general the gross appearance of the lungs in which the lesions result from the repeated dosage with bacilli of a low degree of virulence is identical with the lesion of acute miliary tuberculosis.

In Guinea-pigs VII and XI the lung tubercles on microscopic examination are composed of cells representing two distinct types of lesions. In one the tubercles are made up of polymorphonuclear leucocytes; in the other they are composed chiefly of epithelioid cells. In Guinea-pig XI the remarkable feature of the lesion is that there is not the slightest inflammatory reaction about the tubercles or elsewhere in the lung tissues.

Guinea-pig VI represents the exudative type of lesion. Here the lungs contain areas composed of polymorphonuclear leucocytes, fibrin and desquamated cells. These acute foci are often composed of broken and fragmented cells. The acute lesion of a less severe type presents the same focal character but differs in that the individual cells are well preserved. Thus miliary abscesses occur more commonly in the peribronchial tissues usually about a blood vessel. Others are found near the periphery of a lobe, involving there a limited number of the alveoli. The older foci occasionally show invasion with eosinophiles and lymphoid cells. There is little or no tendency for the surrounding tissue to proliferate even in the more advanced lesions. The tendency is to enlarge by constant accumulation of polymorphonuclear leucocytes and by means of fusion with neighboring foci.

These tubercles are scattered throughout the lung parenchyma. Some of them are just beneath the pleura while others are in the peribronchial tissue. They vary in size from a focus which involves less than half a dozen alveoli to those including many air sacs. (See Plate XVI, Fig. 13.) In no case was there detected a zone of inflammatory reaction. The tubercles, whether early or advanced, are composed of closely packed epithelioid cells filling and entirely obliterating the alveolar meshwork of a given area. In Guinea-pig VII the absence of necrosis associated with the lesions was remarkable, and was probably referable to the early stage of the process and partly to the low grade of virulence of the culture.

This picture of discrete tubercles without necrosis is not uncommon in guinea-pigs infested with tubercle bacilli of low virulence. The necrosis in tuberculosis seems to occur earlier with the more virulent culture. The picture presented in Guinea-pig VII was analogous to that obtained in tuberculosis with bacilli of moderate virulence. On the other hand highly virulent tubercle bacilli produce pulmonary lesions analogous to those produced by the glanders bacilli of moderate virulence. In both, the lesions contain necrosis without the characteristic fragmentation of cell nuclei and formation of chromatin masses.

Giant cells are frequently a part of the lung tubercles. The

lesions in the lungs of Guinea-pig XI did not contain giant cells, while in Guinea-pig VII giant cells were numerous.

The tubercles in Guinea-pig XI, though distinctly proliferative, are more acute than the lesions found in the lungs in Guinea-pig VII where giant cells are numerous. It would seem that the production of giant cells occurs only under certain conditions of specific stimulation. This occurrence of the giant cell after a certain attenuation of the bacilli was noted throughout the work. The culture for the first year after isolation failed to produce proliferation of the invaded tissues; the lesion was invariably inflammatory. However, as the culture generally lost in virulence the inflammatory type of lesion lessened in severity until finally the culture produced only the proliferative lesion. This gradual fall of virulence with a corresponding change in the character and nature of the lesion is of significance.

Testicles.—Here as in other organs the lesions are the acute exudative and the chronic proliferative. In either case the process begins in the tunica vaginalis as discrete foci and spreads towards the skin and the testicle.

The acute focus begins as a collection of polymorphonuclear cells which either undergo rapid disintegration or remain intact, depending upon the virulence of the culture.

The proliferative lesion following the injection of bacilli of low virulence is striking. The tunica propria is studded with discrete and conglomerate tubercles composed of closely packed epithelioid cells and two or more giant cells (Plate XVI, Fig. 16). In the tunic the tubercles are more numerous than in any other organ and tend to coalesce and form large irregular areas which later undergo necrosis. The young tubercles, however, show no evidence of necrosis (see Plate XVI, Fig. 17).

The periphery of the tubercle often shows numerous lymphoid cells. As a rule multinuclear cells occur in the outer zone though often they are situated at the center around which are grouped the epithelioid cells. Some of the giant cells contain thirty or more nuclei, arranged either about the periphery or heaped up in the center of the cell. The nuclei are ovoid and vesicular and stain light blue. In some sections as many as six giant cells can be distinguished lying together in close proximity to one another; often they are adjacent to a tubercle. In sections of the tunica vaginalis in Guinea-pig VII there are as many as nine separate tubercles in a single field under the low power of the microscope. It is not uncommon to find associated with one tubercle six or more giant cells (see Plate XVI, Fig. 17).

The giant cells in relation to the situation of the nuclei may be described in three groups: first, the cell with nuclei arranged centrally; second, the cell with

nuclei situated around the periphery, and third, the cell with nuclei at one or both poles. The giant cell of the glanders tubercle often contains bacilli.

It may be here stated that the glanders bacilli in the tissues differ somewhat in morphology from those of artificial growth. In stained sections the bacilli are more elongated and delicate and contain clear spaces along the sides.

The focal lesions in the tunica vaginalis occur usually in colonies, whether acute or chronic. Beyond the colony of tubercles the tissue is greatly thickened by a fibrous new growth rich in cells. This fibrous cell proliferation often forms a dense zone about the tubercles. In places the connective tissue extends in between the seminiferous tubercles and contains foci of lymphoid and plasma cells. Again the connective tissue invades the muscle fibers which in turn undergo degeneration. Usually there is an active proliferation occurring in the tissue far removed from the seat of the acute inflammation, which is due in part at least to the diffused glanders toxin.

Heart.—Lesions in the heart muscle rarely result from the glanders infection. In two animals where the living bacilli were injected important myocardial changes occurred. Both cases were those of guinea-pigs which had received repeated doses of the attenuated culture over a period of three weeks. The killed bacilli injected intravenously in small and repeated doses sometimes give rise to a marked fatty degeneration of the myocardial fibers in the rabbit.

The lesions are of two types: (1) a fatty change in the muscle fibers which at first is patchy and later becomes more or less diffuse in distribution, and (2) collections of lymphoid and plasma cells which later lead to a fibrous tissue new growth and give rise to definite areas of chronic fibrous myocarditis.

Sections of heart muscle fixed in formalin and stained for fat often show the muscle fibers filled with fat in the form of small globules and fine particles. In some areas the fatty change is diffuse, in others it is slight, while in certain fields only isolated fibers show this degeneration. In addition to the early degeneration of the muscle fibers there are small areas in which the muscle fibers have been replaced by fibrous tissue. Some of these areas are infiltrated with lymphoid and plasma cells, while other areas show only dense fibrous tissue.

Vessels.—The vascular lesions occurring in experimental glanders have been described in detail in a former contribution by one of us (Duval[7]). Babes[8] also mentions the occurrence of vascular changes in glanders in horses.

The lesions are of three general types, (1) the exudative, (2) the proliferative and (3) the degenerative. This grouping of the

[7] *Jour. of Exper. Med.*, 1907, ix, 241.

[8] *Zeit. f. Hyg.*, 1902, xxxix, 217.

lesions corresponds closely with the degree of virulence of the culture employed. In general it can be stated that the highly virulent culture produces vascular lesions of an acute inflammatory type, the culture of moderate virulence produces the proliferative changes, while the degenerative changes result from either the proliferative or exudative lesion.

The vascular changes are focal in distribution. As in other organs the acute lesion is an aggregation of polynuclear cells. They occur in the subendothelial layer of the intima especially in vessels of the peripheral system (Plate XV, Fig. 12). Thrombosis is a frequent lesion in the veins. The pericapillary "round cell" infiltration commonly occurs in the affected areas. Focal collections of eosinophiles in the vessel wall are occasionally met with.

Lymph Glands.—The lymphatic glands in the animals infected with the highly virulent culture are always enlarged and sometimes contain areas of suppuration, especially the lymphatic glands of the inguinal and axillary regions after subcutaneous inoculation, and those of the mesentery and the mediastinum after intraperitoneal injections. In the chronic cases the glands are enlarged and firm, and often contain well-defined areas of caseation.

The acute lesion consists essentially of collections of polynuclear leucocytes, masses of fibrin and disintegrated cells. Here, as in other organs, the more severe of the acute lesions consist of a focus of large and small globular masses of fragmented cells mingled with much indistinguishable material and a few well preserved polynuclear cells. The less acute lesion shows no disintegration of the cells but focal areas composed chiefly of polynuclear cells and fibrin. These acute areas occur in no especial situation. Sometimes they are within or bordering upon the follicles; at other times they occur in the large sinuses, especially in the efferent blood spaces.

The proliferative lesion does not differ in any essential feature from the chronic lesion described for other organs. It is largely composed of the so-called epithelioid cells which form well defined tubercles. Giant cells are found frequently in the proliferative areas of the lymph nodes and often contain the glanders bacilli.

In addition to the proliferative lesions the centers of the follicles contain areas of swollen and degenerated phagocytic cells. These cells often contain fat globules, red blood cells, blood pigment and cell nuclei. The areas of phagocytic cells stain intensely with Scharlach R. and appear in marked contrast to the surrounding deep blue stained cells of the follicle.

Though normally there are numbers of phagocytic cells scattered throughout the lymph nodes of the guinea-pig, in chronic glanders infection they are markedly increased and are especially numerous in the efferent blood sinuses.

Adrenals.—The adrenals are less often the seat of lesion than any other organ, even in animals dying of an acute infection. Only in seven out of forty cases infected with the highly virulent culture were there lesions in the adrenals.

The acute lesions occur both in the cortex and in the medulla and consist essentially of polynuclear collections in which the cells are either fragmented or well preserved. The parenchymal cells about the acute foci are swollen, vacuolated and granular. In some cells there can be demonstrated an occasional polynuclear cell and fibrin threads. The adrenal cells could not be distinguished in the more acute lesions as the degenerative products in the form of large irregular shaped chromatin masses were too abundant. However, in the less severe lesions with perfectly preserved polynuclear cells the necrotic adrenal cells were easily recognized. The adrenal cells within the lesion and those immediately bordering upon the lesion show extremely interesting changes. Many of the adrenal cells for a considerable distance away from the lesion contain mitotic figures. The attempt at division on the part of the parenchymal cells is more manifest and the dividing cells more numerous as the lesion becomes proliferative. Adrenal cells in mitosis are not observed in association with the acute inflammatory type of lesion.

It is interesting to note that there often occurs in the proliferative lesion a central island of adrenal cells enclosed by a well defined broad band of epithelioid cells (see Plate XV, Fig. 10). These "locked off" adrenal cells within the lesion stain intensely with eosin and are often without nuclei, though the general shape of the cell is well preserved. The cell is swollen and the protoplasm contains an excess of fat as demonstrated with the Sharlach R. stain. These areas of necrotic adrenal cells when stained with eosin-methylene-blue present the same general picture as the focal necrosis of the liver parenchyma. Mitotic figures do not appear in the adrenal cells of these areas. The cells immediately surrounding the lesion are also swollen and stained deeply with eosin but to a less degree than the central mass of cells within the lesion. Karyokinetic figures occur only in the adrenal cells neighboring upon the glanders tubercles, thus indicating an attempt at reproduction due to the action of the glanders toxin.

Omentum.—In the acute infections, the omentum is shrivelled up and bathed in a fibrino-purulent exudate.

Microscopically the acute lesions appear rather as definite foci in which the cells are broken and fragmented, or as closely packed collections of polynuclear cells in the meshes of a dense fibrin network.

In the chronic infections the omentum is curled, greatly thickened and contains nodules, many of which are caseous. The microscopic sections show a dense new growth of connective tissue in which are numerous tubercles. In places the fat cells have been entirely replaced by fibrous tissue. There are definite foci of epithelioid cells and areas of necrosis. Giant cells are numerous in the chronic lesions. In this type of lesion many of the vessels are obliterated by old and recent thrombi. There occur definite peri-vascular collections of lymphoid and plasma cells and eosinophiles.

The chronic type of lesion does not differ in any particular from that seen in certain forms of tuberculosis.

Pancreas.—This organ is very seldom the seat of lesion and then only by direct extension of the lesion from the mesentery or neighboring tissues. The lesions are acute and chronic. The acute changes occur in the stroma, only rarely involving the glandular tissue. The chronic lesions are represented by an increase in the connective tissue in scattered areas about the periphery of the lobules and by focal areas of epithelioid cells. The lesion of glanders here differs in no essential from that in other organs.

Intestines.—Lesions of the intestine occurred in only two animals of the whole series. In one the foci were in the large intestine and involved the serous and muscular coats. The lesion consisted of solitary tubercles of the proliferative type and undoubtedly extended directly from the peritoneum. In the other case the lesions consisted of ulcers with undermined edges. Microscopically these ulcerations involved the mucosa and the submucosa and contained necrotic areas about which were numbers of large phagocytic cells, epithelioid cells and polymorphonuclear leucocytes.

Spleen.—The spleen both in the rabbit and the guinea-pig is frequently the site of histological change. Discrete grayish-white areas result from the inoculation of the virulent culture. In this organ as in the others there are two distinct kinds of lesions, the acute inflammatory, resulting from the virulent culture and the chronic proliferative following the inoculation with a culture of low virulence.

The acute inflammatory lesions are of two general types. The first consists chiefly of circumscribed areas of fragmented polymorphonuclear cells and detritus characterized by masses staining dark blue. The second grade of lesion consists chiefly of polymorphonuclear leucocytes that show no tendency to break up and give rise to the characteristic chromatin masses above described. These lesions in addition contain large masses of fibrin which is arranged in a dense meshwork through the lesion.

The spleen sections of Guinea-pig XV of the intraorbital series of inoculations contain irregular masses of fibrin in the form of thrombi plugging the capillaries (see Plate XV, Fig. 11). The animal had recovered from the acute infection. The eyeball had been completely destroyed and the tissues of the orbit were greatly thickened presenting a large sloughing caseating mass.

The proliferative type of lesion occurs as well defined tubercles scattered throughout the section, though the site of predilection seems to be in connection

with the Malpighian bodies. Hence the lesion apparently begins just outside the follicle, gradually increases in size and invades by extension. These lesions are best developed in animals killed two or three weeks after inoculation with bacilli of a moderate degree of virulence.

Giant cells occur frequently and are either associated with the tubercles or are in their immediate neighborhood (see Plate XV, Fig. 9). Many of the tubercles are formed about the giant cells. These multinucleated elements contain from four to twenty nuclei arranged either centrally or peripherally within the cell protoplasm and are phagocytic for the glanders bacilli (see Plate XIV, Fig. 6).

The tubercles range from one third to one half the size of the Malpighian body and are composed chiefly of large cells having an ovoid lightly-stained vesicular nucleus. Lymphoid and plasma cells are found in the periphery of the tubercle. The contrast between the foci composed of these large proliferated cells and the cells of the Malpighian body with the eosin-methylene-blue is striking. The dark blue nuclei of the cells constituting the Malpighian body are in marked contrast to the light blue nuclei of the epithelioid cells, the nuclei being embedded in a protoplasmic matrix staining pink. These areas of epithelioid and giant cells are histologically similar to the miliary tubercle. They increase in size and coalesce with neighboring tubercles to form large conglomerate areas, which later undergo necrosis.

In addition to the glanders tubercle the blood sinuses contain large numbers of phagocytic cells containing pigment, fragmented nuclei and red blood cells. In the section of a spleen of a rabbit with subacute focal lesions the phagocytic cells often contain from three to six ovoid vesicular nuclei. Occasionally a Malpighian body contains multinucleated phagocytes undergoing fatty and hyaline change; other Malpighian bodies contain in well defined foci phagocytes in which the fat is admirably demonstrated with the Sharlach R. stain.

Giant cells and epithelioid cells are more numerous in the spleen of animals killed two or three weeks ofter inoculation. Here they occur in the blood channels, either free in the lumen or attached to the endothelium. The attached cells are swollen to many times their normal size, often causing partial or complete occlusion of the vessel (see Plate XV, Fig. 8). Not infrequently these swollen attached cells contain in addition to separate nuclei, well defined mitotic figures. Animals inoculated with bacilli of low grade of virulence afford an excellent opportunity to follow the development and function of these multinucleated elements.

Kidney.—This organ with the exception of the pancreas and adrenal is less commonly the seat of lesion than any other. In the cases of acute infection following the injection of highly virulent bacilli, albuminous and hyaline degeneration of the tubular epithelium usually occurs. The glanders nodules, so common in other organs, are rarely met with in the kidney. Occasionally miliary abscesses are encountered.

Intravenous injection sometimes gives rise to a peculiar type of

kidney lesion in which the organ presents the appearance of an acute hæmorrhagic nephritis. The following autopsy protocol of Rabbit XIX well illustrates the gross appearance of this type of lesion.

Rabbit XIX.—A full-grown female rabbit was inoculated intravenously with 1 c.c. of a twenty-four hour agar slant growth suspended in 6 c.c. of bouillon. The animal at the time of inoculation was nursing her young. Three other rabbits, one male, one pregnant, and one non-pregnant female, were inoculated at the same time with equal amounts of the culture under identical conditions. The animal with young developed a severe grade of acute glanders and was killed three days following the inoculation. The animal was very ill. A thick, purulent discharge ran from her nose and conjunctivæ. She was constantly wheezing and presented the typical appearance of snuffles. At autopsy the mucous membrane of the conjunctivæ and naso-pharynx was swollen, deeply congested and bathed in a sero-purulent exudate, which on examination was found to contain numerous bacilli of the morphology of *B. mallei.* A pure culture of this microörganism was recovered by plating the material from both the nose and pharynx. The veins of the ear about the site of inoculation are hard and in places nodular. On section they contain organized thrombi. The mammary glands are swollen, tense and on section markedly hæmorrhagic. The peritoneal cavity appears normal. The kidneys are twice their normal size and presented innumerable discrete pin-point and larger petechial hæmorrhages over the surface. These hæmorrhagic areas were all the more striking owing to the pale grayish-red color of the cortex. On stripping away the capsule, the cortical hæmorrhages remain attached to the cortex and project above the surface as firm coagulæ. On section these hæmorrhagic areas extend all through the organs, parallel to the surface and in more or less beaded streaks which apparently correspond to the general direction of the tubules.

The adrenals are swollen and deeply congested. The liver presents grayish-white irregular areas which are plainly visible beneath the capsule. Microscopically these areas are those of focal necrosis. The lymph nodes throughout the body are enlarged. The other organs are negative.

Sections of the kidney fixed in Zenker's fluid and stained with eosin-methylene-blue show the renal tubules filled with coagulated blood stained serum. The microscopic examination of sections under the low power show the tubules everywhere greatly dilated and the lumen filled with a light material staining pale pink in striking contrast to the deep distinct blue color of the tubular epithelium. The same material staining pink is also present in the capillary spaces of the glomeruli. On closer study, this material is found to be composed of altered blood cells and serum and occurs in at least three different forms: (1) finely granular reticulum, (2) homogeneous pink coagulum, and (3) large and small spherical globules. In the glomerular spaces and convoluted tubules, this material occurs as a structureless homogeneous mass; in the straight tubules it appears granular and finally as spherical globules.

The tubular epithelium, though compressed, is for the most part well stained and everywhere intact.

The deep red hæmorrhagic points projecting above the surface are firm coagula. In addition to the altered blood filling the tubules there is still another material, epithelial in origin, which is met with in three forms. (1) Complete separation of the intact lining epithelial cells forms casts which occupy the central parts of the lumen. These cells, from the absence of nuclei and their intense eosin staining, are unquestionably necrotic though their shape is well preserved and it would seem as though they were destroyed in situ and later fell away in columns from their basal attachment. (2) The tubules contain material composed of granular and globular degeneration products of epithelial protoplasm. (3) Tubular epithelium free in the lumen shows early granular degeneration with the nucleus still intact, and the cells are also seen converted into small and large globular masses staining a deep purple red.

In the collecting tubules the epithelium is often swollen to three times its normal size. Each cell, though markedly vacuolated, preserves its shape. Often the free edges meet and completely obliterate the lumen. The epithelium of the tubules of the pyramids, in addition to this swelling and vacuolation of the protoplasm, undergoes proliferation and in places the cells are heaped up six or eight deep and many contain mitotic figures.

The glomeruli are enormously swollen and filled with altered blood in the form of material staining pale pink. Many glomeruli show distinct masses of fibrin occluding the capillary space. Occasionally the lining cells of the glomerular capillaries are swollen and contain mitotic figures. In a few glomeruli the capillaries are filled with polynuclear cells.

The connective tissue stroma of the kidney, even in the acute infections, rarely shows change except that it contains occasionally foci of lymphoid cells and a few scattered eosinophiles.

The peculiar material described by Wright as filling up the kidney tubules of certain animals dying of acute glanders infection undoubtedly had its analogy in Rabbit XIX, although he does not describe the gross appearance of the kidneys. From the above description of the gross and microscopic appearance of the kidneys of Rabbit XIX it will be readily seen that this peculiar material is coagulated blood stained serum.

DISCUSSION.

From the foregoing description of the histological lesions of experimental glanders it will be seen that the glanders bacillus and its toxin produce three distinct types of change in the tissues, exudative, proliferative and degenerative, the determining factor being the degree of virulence of the culture. Highly virulent bacilli produce exudative changes; bacilli of lowered virulence produce proliferative changes. The exudative type includes acute lesions; the proliferative type includes chronic lesions, the degree

of chronicity varying directly with the attenuation of the culture. The degenerative type is always secondary either to the exudative or proliferative lesion.

The exudative lesions fall naturally into two groups, in accordance with the degree of virulence of the culture employed.

In the first group, produced by the most virulent cultures, the tissue cells and the rapidly invading polymorphonuclear leucocytes are broken up by the virulent toxin, and the broken down nuclei of these cells form the degenerative "chromatin masses." These chromatin masses were described by Wright as the chief characteristic of the glanders nodule. Wright's observations were limited to the effects of cultures of high virulence. He produced experimentally the lesions only of this first group, *i. e.*, lesions of the exudative type.

In the second group, produced by a less virulent culture, the lesion consists of closely packed collections of well preserved polynuclear leucocytes and fibrin. There is here an entire absence of the destructive action of the more virulent bacilli, which alone is evidence that the lesion varies with the degree of virulence of the culture.

The degenerative lesions occur for the most part in the liver, adrenals, heart and arteries. In the liver the degeneration occurs as a focal necrosis involving either single widely separated parenchymal cells, small groups of cells, or larger groups of cells comprising the greater part of a lobule. This cell necrosis is caused by the large multinuclear cells and by thrombi of fibrin. Mallory[9] states that thrombi of fibrin form in the capillaries under at least two conditions, viz., adjoining necrotic liver cells and around endothelial or other cells which have undergone necrosis within the capillaries.

The adrenal degeneration also occurs in the parenchyma either in single cells or in large and small groups of cells scattered throughout the cortex and medulla. It is caused partly by direct toxic action and partly by interference with nutrition resulting from the encircling epithelioid cells; the latter leads to the isolation of groups of adrenal cells.

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[9] *Jour. of Exper. Med.*, 1895, iii, 611; *Jour. of Med. Research*, 1901, i, 264.

Degeneration of the heart muscle undoubtedly occurs as the result of repeated doses of the glanders poison. It is exhibited by the occurrence of fine droplets of fat within the muscle fibers. Some of these degenerated fibers are later replaced by newly formed connective tissue, giving rise to definite areas of chronic fibrous myocarditis.

The degenerative vascular lesions have been described by one of us in a former paper. Briefly there is a fatty change in the muscle fibers of the inner zone of the media secondary to primary intimal proliferation.

The main object of this series of experiments was to determine whether or not the glanders bacillus, under certain conditions, produced lesions resembling histologically those of tuberculosis and if such lesions existed, to determine the analogy between the glanders tubercle and the miliary tubercle, especially with regard to their histogenesis. The inoculations were made with glanders cultures of three degrees of virulence with the hope that the bacilli of moderate or low virulence might exert an irritative rather than the destructive action so characteristic of the highly virulent bacilli, and instead of causing exudation give rise to proliferation.

It became apparent that as the bacilli became less virulent the character of the lesion approached more closely that of tuberculosis. A gradation in severity of the lesion could be followed from the acute to the chronic changes, *i. e.,* from exudation to proliferation, until finally the type of lesion became analogous to that of tuberculosis.

The histogenesis of the tubercle of glanders is primarily a proliferation of the invaded tissues with the production of the so-called epithelioid cells, associated with giant cells (see Plate XIII, Fig. 1).

When Baumgarten stated that the primary effect of the glanders bacillus on the tissue was a production of epithelioid cells and that the glanders tubercle was essentially the same as the miliary tubercle, he was correct, but he failed to recognize the special conditions under which Bacillus mallei gives rise to the proliferative type of lesion and he did not observe that the organism produced more commonly the acute inflammatory lesion.

The glanders tubercle is best studied in the spleen, lungs, testes

and lymph nodes. It is composed almost entirely of closely packed epithelioid cells; its outer limits often show numerous invading lymphoid cells, and a few polynuclear leucocytes penetrating toward the center. The giant cells are usually arranged about the periphery, although not infrequently they occur in the central part of the tubercle (see Plate XV, Fig. 9).

The histology of the glanders tubercle is essentially that of the miliary tubercle and varies with the virulence of the infection; but the variation is the reverse of that which occurs with the lesion of tuberculosis. In the early tubercles of acute miliary tuberculosis produced by highly virulent tubercle bacilli there is an absence of giant cells and necrosis. In the early tubercles produced by the attenuated bacillus of glanders the giant cell is absent and there is only rarely necrosis. The lesion of the highly virulent tubercle bacillus corresponds with the lesion of the attenuated glanders bacillus.

The epithelioid cell marks the transition from the acute exudative to the chronic proliferative lesion in which it is the essential element. Even in the subacute lesion it occurs frequently in the inflammatory zone. It constitutes the entire formation of the early tubercle. In the lungs frequently occur discrete tubercles composed entirely of epithelioid aggregations without evidence of surounding inflammatory reaction. The origin of the epithelioid cell is still doubtful. It would seem to originate from the connective tissue cell. The fact, however, that it never becomes phagocytic either for bacteria or for cell products in the glanders tubercle is rather against its endothelial origin because the endothelial cell is phagocytic under the most varied conditions.

In general the epithelioid cells are large with pale staining ovoid vesicular nuclei. In morphology and staining reaction, and also in their focal arrangement they are identical with the epithelioid cells of the tuberculous lesion; indeed, under the microscope the glanders tubercle is indistinguishable from that of miliary tuberculosis.

The giant cells of glanders are found in the tubercles of the proliferative lesion, in the capillaries and in the lymph spaces at some distance from and entirely outside of the lesion. As in tuberculosis they are of both the tuberculous and "foreign-body"

type; indeed these two types are often associated in the same lesion. The giant cells are especially numerous in lesions of the testes, lungs, lymph nodes and spleen. The lesions in the spleen afford an excellent field in which to trace the evolution of the giant cell.

The first sign of a reaction is the swelling of isolated endothelial cells of the blood or lymph channels to twice or three times their Such a cell bulges far into the lumen although still attached to the vessel wall gradually becoming larger and encroaching upon the lumen. This increase in the size of the endothelial cell is due to the proliferation of the nucleus which first undergoes karyokinetic division. The nuclei resulting then divide directly, this process continuing until the cell becomes dilated to enormous proportions. Such a cell bulges for into the lumen although still attached to the vessel wall. The adjacent endothelial cells may not show the slightest change; this fact is remarkable as there seems to be no explanation for the peculiar activity in isolated cells. Either in this stage or in the later detached stage the cell may be so large as to occlude and even distend one portion of the lumen of the vessel while the adjacent portions maintain their normal size. The detachment of the endothelial cell occurs usually before the proliferation of the nuclei is complete, the distended cell containing twenty or more nuclei arranged in rouleaux about the periphery.

In one section it was possible to follow the endothelial cell through various transitional stages to the formation of large multinucleated elements. This section was cut from the spleen of a guinea-pig killed two weeks after inoculation with a glanders culture of low virulence. The bacilli were stained in the lesion and recovered from the organs.

In addition to the primary swelling of the attached endothelial cell it is not uncommon to find in the small blood vessels large cells from twenty to thirty microns in diameter whose limiting membrane is sharply outlined and whose nuclei present every evidence of direct division. Such cells have three or four separate nuclei arranged more or less in a row and two or more nuclear masses apparently undergoing direct division (see Plate XIV, Fig. 7). These nuclear masses may consist of two bulbous ends connected by a narrow bridge of nuclear substance. In one bulb which is twice

the size of the other, there is evidence of division into separate compartments by means of fine threads. In each compartment there is a deep staining granule. The other bulb is composed of one compartment only and this contains a single large granule; the whole mass appears as a distinct nucleus except for the bridge still holding it to the smaller mass. One can easily perceive that a section though a plane above or below this joining bridge would give the effect of separate nuclei (see Plate XIV, Fig. 5).

In the study of other giant cells in the same section the evidence of direct nuclear division is even more striking. In one cell the dividing nuclear masses are connected by narrow bands and the whole arranged in a semicircular manner about the periphery of the cell. One end of this nuclear chain consists of smaller masses more nearly perfected and separated than the masses of the outer end of the chain where they are larger and less distinctly divided off (see Plate XIV, Fig. 4). Still another stage in the development of the giant cell is one in which the nucleus is apparently beginning to divide. In such a cell the nuclear mass is situated centrally and is composed of three or more vesicular bulbous swellings, each having the internal structure of a separate nucleus and connected in a circular manner one with the other by means of narrow bands of nuclear substance (see Plate XIV, Fig. 3).

This manner of nuclear division continues until the cell is crowded with nuclei. Finally the cells undergo necrosis in a manner characteristic of giant cells. Occasionally these giant cells contain pigments or parts of other cells. In the glanders tubercle they frequently contain numbers of glanders bacilli (see Plate XIV, Fig. 6).

The study of the various lesions produced by B. Mallei suggests that stimulation of the endothelium by the weak toxin is the deciding factor in the production of the glanders giant cell. It is well recognized that the endothelial phagocyte is capable of undergoing a marked increase in cell extent whether the nucleus divides or not. Even when the cell has migrated and is no longer a part of the vessel wall its protoplasm is capable of enormous distension, an example of this condition is in the phagocytic endothelial cell of typhoid fever.

While there is no evidence of the fusion of cells in the formation of the giant cell of glanders there is a proof of its evolution from the endothelial cell through nuclear proliferation.

SUMMARY.

The bacillus of glanders may be so modified in virulence as to produce experimentally lesions differing widely in their histological features.

The highly virulent culture causes primary necrosis and disintegration of the tissue followed by the invasion of the injured area by polymorphonuclear leucocytes. The bacilli of moderate virulence give rise to a primary lesion of an acute inflammatory nature in which the cells show no evidence of necrosis or disintegration. The attenuated bacilli produce primary tissue proliferation with the formation of epithelioid and giant cells.

There is every grade of lesion between the acute exudative and the chronic proliferative depending upon the toxicity of the cultures.

The glanders lesion whether exudative or proliferative is focal in character.

The strong toxins of the glanders bacilli cause degeneration or necrosis of cells and exudation, while the dilute and weak toxins produce proliferation.

The giant cell of glanders undoubtedly originates from the endothelial cell of the blood and lymph channels and is formed by division of the nucleus of the endothelial cell and not by cell fusion.

Histologically the lesion of glanders resulting from the culture of a low degree of virulence is proliferative and is analogous to tuberculosis; the lesions are focal and bear an intimate relation to the glanders bacillus.

DESCRIPTION OF PLATES.

PLATE XIII.

FIG. 1. A glanders tubercle. The proliferative type of lesion which results from the invasion of the tissues by the attenuated bacilli.

FIG. 2. Section of the rabbit's cornea stained by a special method to demonstrate the glanders bacilli in the tissues. The section also shows the early collection of large acidophilic granule cells about the injured area.

PLATE XIV.

FIGS. 3, 4, 5. Represent the various stages in the nuclear division of the endothelial cell in the production of the glanders giant cell.

FIG. 6. Shows a typical giant cell containing glanders bacilli.

FIG. 7. Shows a large multinucleated cell free in the lumen of a capillary.

PLATE XV.

FIG. 8. Section of spleen of the guinea-pig showing (*a*) single swollen endothelial cell of the capillary, (*b*) endothelial cell enormously enlarged and containing many nuclei and still attached to the vessel wall, and (*c*) large giant cell in the lumen of capillary.

FIG. 9. Section of spleen of the guinea-pig showing glanders tubercle, which is composed of epithelioid cells grouped about a central giant cell.

FIG. 10. Section of the rabbit's adrenal showing an area of necrotic adrenal cells surrounded by a dense zone of epithelioid cells.

FIG. 11. Section of the guinea-pig's spleen under low magnification. Note the fibrin thrombi everywhere plugging the blood sinuses.

FIG. 12. Section of the guinea-pig's omentum containing a small artery. Note the focal collection of polynuclear cells between the intima and media.

PLATE XVI.

FIG. 13. Section of lung of rabbit showing a sharply defined "glanders tubercle" near the pleural surface. The tubercle is composed of epithelioid cells, giant cells and necrotic material; and histologically is analogous to the miliary tubercle.

FIG. 14. Early glanders tubercle in the rabbit's cornea. The lesion is focal and composed chiefly of epithelioid cells, which undoubtedly have their origin in the corneal connective tissue cells.

FIG. 15. Section of rabbit's liver showing extensive focal necrosis. Fibrinous thrombi are numerous in the sinusoidal spaces of the affected lobules.

FIG. 16. Section of the tunica vaginalis of the guinea-pig showing multiple glanders foci composed of epithelioid and giant cells.

FIG. 17. Section of the tunica vaginalis of the guinea-pig showing a solitary glanders tubercle which is composed of epithelioid and giant cells, and moderate numbers of polynuclear leucocytes.

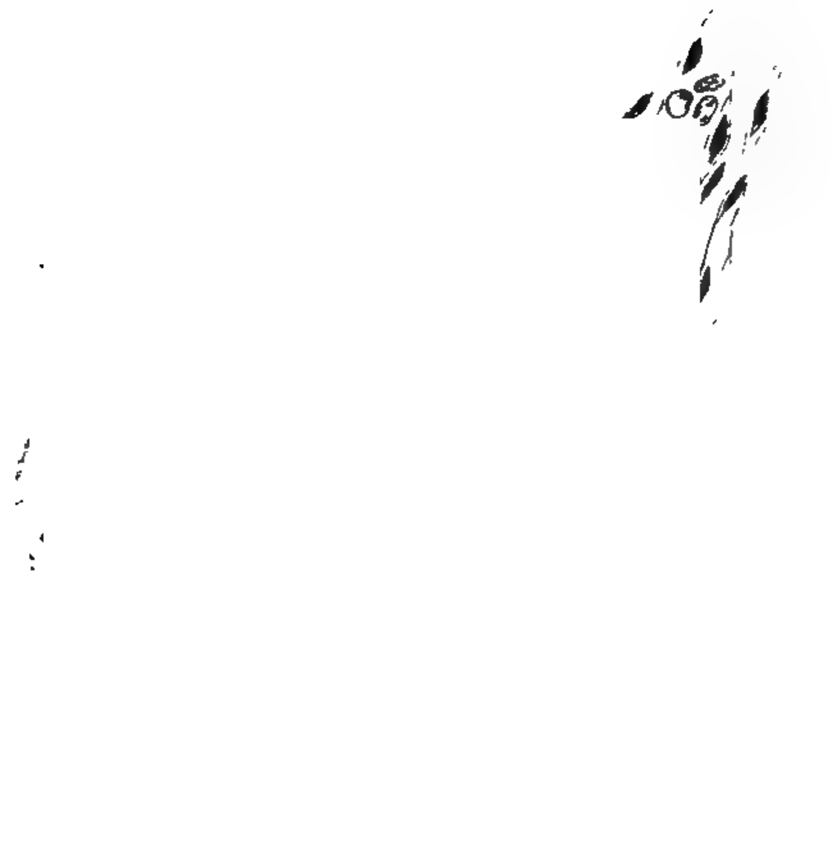

FIG. 1

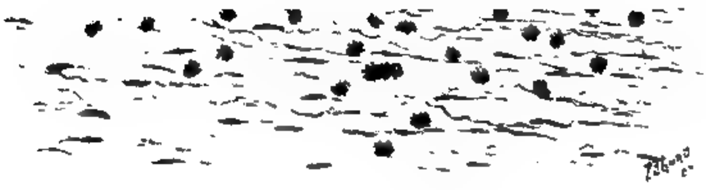

FIG. 2.

Fig. 3.

Fig. 4.

Fig. 5.

Fig. 6.

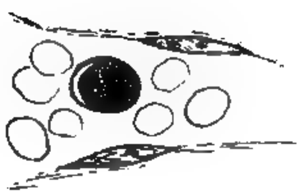

Fig. 7.

FIG. 11.

FIG. 12.

FIG. 16.

FIG. 17.

A METHOD OF DIFFERENTIATING IN SECTIONS OF TISSUE BACTERIA DECOLORIZED BY GRAM'S STAIN.

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A number of methods are given in the text books on histological technique for staining in the tissues bacteria which are decolorized by Gram's method, but owing to the fact that the demonstration of the bacteria is accomplished at the expense of the tissue differentiation they have all proved unsatisfactory.

Good results are only occasionally obtained by the ordinary routine eosin-methylene-blue method (recommended in Mallory and Wright's text book on Pathological Technique) and are too inconstant to be of practical value in staining bacteria in sections. The failure of the method to give constant results does not seem to be the fault of any one step in the technique, therefore attempts to correct or modify this method have been unsuccessful.

In the course of a study of the histological lesions produced by certain bacteria which do not retain the Gram stain, the attempt was made to demonstrate the organisms and at the same time to obtain a clear differentiation of the tissues. The well differentiated cell-picture obtained with the various modifications of Leischman's blood stain (especially that devised by Wright), suggested to me this method, or some modification of it, as a possible means of overcoming the difficulty heretofore encountered. The experiment succeeded beyond all expectation.

The staining solution used is a modification of the Leischman-Wright solution; the essential difference being in the amount of eosin used. The ordinary Leischman stain and the various modifications will not give color differentiation to the tissues. They all stain the tissues uniformly blue with little if any nuclear differentiation so that the bacteria if stained are not distinguishable.

Since the method of preparing this special stain differs somewhat from that described for the various polychrome-staining mixtures, the technique will be given here in detail.

Thoroughly dissolve 0.5 gram of sodium carbonate in 100 c.c. of hot distilled water, and while hot add 1 gram of Grübler's methylene-blue. The mixture is now steamed in an Erlenmeyer flask over a water bath until there is formed on the surface a distinct metallic luster, which usually appears within from twenty to thirty minutes. The solution is now cooled and 900 c.c. of 0.25 per cent. aqueous solution of Grübler's eosin is added slowly, the flask being thoroughly shaken after each addition. Finally a precipitate forms which is collected on a filter paper and while in the thick, moist state is turned out into a dish and dried in the incubator at 55° C. The drying is accomplished in one to two hours. Dissolve one gram of the dry precipitate in 100 c.c. of a 0.5 saturated alcoholic (methyl) solution of eosin. The stain should be made fresh before using. The dried precipitate can be kept as a stock from which the fresh stain is prepared as needed.

Method of Application.—The tissues are cut in sections from 2 to 4 micro-millimeters in thickness and fixed in eighty per cent. methyl alcohol for twenty-four hours and embedded in paraffin. Zenker's fixation also gives good results though it is not as satisfactory as with methyl alcohol. Paraffin sections are cut as thin as possible and fixed on slides in the usual way by means of an albumen solution. The paraffin, Zenker's fluid, and iodine are removed from the tissues by the ordinary method of procedure. The sections are now placed in ninety-five per cent. methyl alcohol until ready to stain.

The slide containing the section is removed from the methyl alcohol and placed in a level position over a small tumbler or slide support and covered evenly with the staining solution, care being used not to run the stain over the edges of the slide. The stain should be added before the methyl alcohol has time to evaporate in order to avoid the drying of the section. Distilled water is now slowly added by means of a dropper, until a metallic sheen forms upon the surface of the stain. This usually occurs when the phenomenon of ebullition ceases. The section is left covered in the diluted stain thirty minutes.

The next step is the differentiation of the tissue which is the most important one in the whole procedure. The excess of stain is first poured off and the slide placed in a dish of distilled water in which it is constantly moved to and fro for from ten to fifteen minutes. The water should be changed two or three times in the course of the process and the section examined from time to time under the low power of the microscope to determine the progress of the differentiation.

The stained section when completely differentiated appears to the naked eye of a distinct purple red color, whereas in the beginning it was of a deep purple blue, varying in degree with the tissue under examination. The microscopic examination shows the nuclei a distinct blue and the intercellular tissues a deep pink. The differentiation in water, particularly when the water is constantly agitated, intensifies the eosin stain of the tissue and cell protoplasm and to some extent tones down the blue stain of the nuclei.

The most satisfactory method of dehydrating and clearing is to first quickly blot off the excess of water by means of absorbent tissue paper (using every care to avoid a drying of the section), then to add a few drops of ninety-five per cent. methyl alcohol followed immediately with xylol. The secret of the whole procedure, after the section is stained and differentiated, is in properly dehydrating without decolorizing the bacteria or taking the eosin stain from the cell protoplasm and intercellular tissues.

Sections dehydrated in alcohol for more than fifteen seconds may be completely decolorized of their eosin. By first removing the excess of water by means of blotting paper a few seconds are sufficient to dehydrate the section.

The summary of the steps is as follows:

1 Fix tissues in methyl alcohol (or Zenker's fluid) and embed in paraffin.

2. Treat sections in the usual way to remove the paraffin (or Zenker's fluid and iodine) and place in 95 per cent. methyl alcohol.

3. Remove slide from methyl alcohol and cover evenly with the staining solution, using care not to run the stain over the edges.

4. Add to the stain-covered slide an equal quantity of distilled water (avoid running the stain over the edges) and allow it to stand in the admixture from 15 to 30 minutes.

5. Pour off excess of stain and differentiate in distilled water for 15 to 30 minutes, constantly keeping the water in motion.

6. Blot off excess of water with tissue paper (do not allow to dry) and dehydrate for a few seconds in 95 per cent. methyl alcohol, or dehydrate and clear in analin oil, first blotting.
7. Clear in xylol.
8. Mount in balsam.

This method not only renders possible the demonstration of bacteria in tissue sections but gives a perfectly satisfactory tissue stain in which the various elements are clearly differentiated. The microscopic picture is even more delicate and distinct in color contrast than that obtained with the eosin-methylene-blue method after Zenker's fixation. The color contrast is especially well marked in sections of the spleen and lymph nodes where the protoplasm of the individual cells is well preserved. Here the various elements can be as readily differentiated by their nuclear and protoplasmic staining reaction as the cells of a blood film. The phagocytic cells with their inclusions are especially striking in color contrast. Blood platelets and fibrin are well stained. The connective and muscle tissues are stained a deep pink color. Sections of the cornea and skin are especially well differentiated by this method of staining. The epithelial cells are colored a distinct robin's-egg blue in contrast to the clear pink staining of the underlying connective tissue. Bacteria take a deep blue-black stain and are readily detected either in the cells or the intercellular substance.

The perfect staining of the various tissues with the differentiation of the chromatin and cell protoplasm renders the method most useful in the demonstration of protozoa, such as the amoeba-coli, in tissues and offers a more certain method of recognizing protozoan-like bodies in the cells and intercellular spaces of the skin in certain contagious diseases.

A STUDY OF THE EFFECT OF INTRAVENOUS INJECTIONS OF SOLUTIONS OF PANCREATIC TISSUE; WITH ESPECIAL REFERENCE TO THE CAUSE OF COLLAPSE IN ACUTE PANCREATITIS.*

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Blood-pressure in disease and especially in its relation to shock and collapse has been the subject of much experimental work. There is a particular phase of this subject dealing with the influence of tissue extracts and products of tissue degeneration in disease that is of special interest. Powerful toxins can be extracted from certain organs and others are formed in the process of auto-digestion or breaking down due to bacterial action.

There is evidence to show that autolysis differs somewhat from the ordinary decomposition of tissue. Biondi[1] believes there are several ferments differing from trypsin at work in self-digestion. They are specific for certain proteid molecules of the nature of globulins and cause the formation of end products rather than of albumoses. Salkowski believes a ferment is set free at time of death. Salkowski, Jacobi and others have shown that the cells of the animal body contain ferments capable of digesting these cells under certain conditions. Schwiening and Jacobi do not consider the ferment identical with trypsin.

Claypon and Schryver[2] have published some experiments on autolysis. They show that there is a latent period of twenty-four hours after extraction before autolysis begins. The authors believe the ferments active in this process do not exist preformed in the cells, but as zymogens. On the liberation of the enzymes autolysis begins. The fact that they found the latent period diminished

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[1] Biondi, *Virchow's Arch.*, 1896, cxliv, 373.

[2] Claypon and Schryver, *Jour. of Physiol.*, 1904, xxxi, 169.

or absent in organs of fasting animals suggests that liberation of those enzymes is a normal mechanism for the intra-vitam utilization of the proteids of the tissues. Even in the earliest stages the maximum amount of degradation products is in the form of the lower molecular compounds.

It has been proved that extracts from certain organs possess a depressing effect on the blood pressure. Osborne and Vincent,[3] and Vincent and Sheen[4] have studied this phenomenon. Vincent and Sheen found that the extracts of nervous tissues produce a marked temporary fall of blood-pressure occurring after section of both vagi and after doses of atropine sufficient to abolish vagus action. They do not believe choline is the active principle of nervous tissue extracts. They found extracts from muscle tissue, kidneys, testes, pancreas, liver, spleen, ovary, lungs, and intestinal mucous membrane produce depressor effects.

Vincent and Cramer[5] showed that normal blood of the ox and rabbit produced frequently as great a fall of blood pressure in cats and rabbits as Halliburton observed when he used pathological blood. It can then be seen that many organs possess substances that are capable of influencing the blood-pressure.

In regard to certain products of decomposition or autolysis Hamburger[6] has found that peptones and albumoses exert on the organism first a narcotic action resembling that of chloroform; second, an anticoagulative effect when injected intravenously; third, an effect on the blood-pressure. In the form of Witte's peptone it produced a fall in pressure. All the ingredients of Witte's peptone with the exception of anti-peptone possess undoubted vaso-dilating properties. Pick and Spiro found it possible to obtain typical albumoses and peptones after cleavage of proteids by trypsin, autolysis, and alkalies, but these authors found that when such products are introduced into the blood no effect was shown on the blood-pressure. They believe active products prepared by acid or pepsin and acid lose their typical action after purification by a method which apparently does not alter their chemical character.

[3] Osborne and Vincent, *Jour. of Physiol.*, 1900, xxv, 283.
[4] Vincent and Sheen, *ibid.*, 1903, xxix, 242.
[5] Vincent and Cramer, *ibid.*, 1903–4, xxx, 143.
[6] Hamburger, *Amer. Jour. of Physiol.*, 1904, xi, 282.

Mendel and Underhill[7] doubt some of these conclusions. They found that all proteose preparations introduced in doses of from 0.3 to 0.5 gram per kilo of body weight into the circulation of dogs produced a fall in arterial pressure, diminished coagulability of the blood, a transitory stage of excitation followed by narcosis, by a degree of immunity, and in a single experiment so far tried by lymphagogic effect.

The collapse so characteristic of acute pancreatitis has been the subject of considerable study of late. Several have offered explanations. Opie[8] believes some toxin is the cause; Guleke[9] says it is due to trypsin; Doberauer[10] believes it is caused by some toxin derived from the pancreatic tissue. Involvement of the cæliac plexus is advanced by others as the cause. That the stretching and irritation of the sensitive peritoneum, and involvement of the cæliac plexus are important contributing factors seems very probable in view of the exceedingly severe pain seen in the disease. Some lowering of blood-pressure can be produced by irritating these parts experimentally. This was done on four dogs using mechanical irritation and three per cent. nitric acid. Although the blood-pressure fell and there was considerable disturbance in respiration and pulse tracings, there was not sufficient evidence to indicate that the collapse could be explained on the basis of irritation of the peritoneum or involvement of the cæliac plexus alone.

We have in the products of tissue decomposition of the pancreas substances capable of producing marked toxic symptoms and fall in blood-pressure. The general opinion seems to favor a toxin as being the cause of this collapse or at least as being the main cause. That the pancreas will undergo a very rapid autolysis is well known. There is marked tendency for a small injury to spread, there is breaking down of tissue and more or less necrotic material tends to form. In many cases the peritoneal cavity is partially or completely filled with fluid. That the conditions for absorption of toxic substances are favorable is evident.

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[7] Mendel and Underhill, *Amer. Jour. of Physiol.*, 1902–3, viii, 377.
[8] Opie, Diseases of the Pancreas, Philadelphia, 1903.
[9] Guleke, *Archiv. f. klin. Chir.*, 1905–6, lxxviii, 845.
[10] Doberauer, *Beiträge z. klin. Chir.*, 1906, xlviii, 456.

It has been suggested that the glycerine formed in the production of fat necrosis is responsible at least in part for the collapse. However after repeated trials on dogs and rats obtaining no satisfactory results I came to the conclusion that it played an unimportant part.

As already stated, trypsin has been advanced as a possible cause of the collapse. That it has a depressing effect is obvious from the results obtained. But the effect is not as pronounced as with peptone and other tissue extracts of the pancreas, and is more transient. So experimentally at least I do not regard trypsin as important. Cathcart[11] has found that normal serum has an antitryptic action which is associated with the albumin fraction. It was found in all varieties of sera examined by the author and is destroyed by exposure to 55° C. for one half hour. Globulins do not possess this action.

In acute pancreatitis the toxin or toxins causing the marked collapse are probably derived from the broken down pancreatic tissue, disintegration being brought about by the action of bile salts in case of retrojection of bile, and possibly through the action of ferments contained in the gland itself. Bacterial action also would readily lead to the formation of necrotic material in this gland, so readily broken down. Peptones and albumoses would be the early substances formed, later on aromatic compounds as indol and skatol, and certain phenyl-compounds would appear. Finally, according to Levene,[12] the amino-endproducts are in case of self-digestion of the pancreas, alanin, amino-valerianic acid, leucin, glutaminic acid and aspartic acids, tyrosin and phenylalanin; α-pyrrolidin-carbonic acid could not be established with certainty.

That the degradation of tissue is rapid even in cases of pure autolysis is seen from Hamburger's experiments. He found that often the increase of nitrogenous extractives in case of the liver after an incubation of twenty-four hours amounted to more than fifty per cent. of the total.

Peptone is an early product formed in the breaking up of cellular substances either through autolysis or bacterial action. This was injected into the femoral vein of three dogs, causing a fall slightly

[11] Cathcart, *Jour. of Physiol.*, 1904–5, xxxii, 299.
[12] Levene, *Amer. Jour. of Physiol.*, 1904, xii, 276.

in excess of that produced by trypsin and a little more prolonged.

Experiments.—To test the effect of pancreatic extracts on the blood-pressure the following experiments were undertaken. These can be divided into two classes. The testing of extracts (1) giving the biuret reaction, (2) those not giving the biuret reaction. The extracts were prepared as a watery solution of fresh ground pancreas obtained from dogs. Ten dogs were used for the experiment. A canula was inserted into the left carotid artery and tracings taken on a slowly revolving drum. The injections were made into the femoral vein in most experiments.

Injections of pancreatic extracts giving the biuret reaction had a marked effect on the blood-pressure. The rapidity and extent of the fall was more pronounced than after the injection of either trypsin or peptone, or after mechanical irritation. Doses from 2.5 c.c. to 2 c.mm. were employed and were always followed by a rapid fall of about 20 mm. in the mercury column. Although there was a considerable difference in the amounts injected at different times, the difference in response was not as great as might have been expected. The results obtained showed that there was present in the extracts a depressor substance of considerable toxicity. The trypsin and peptone present had possibly some effect, but the fall in pressure was considerably in excess of that produced by trypsin or peptones used alone; therefore there must be present some other substance or substances in the gland tissue which is more powerful than those mentioned. The same results were obtained when one or both vagi were cut.

The inoculation of pancreatic extract not giving the biuret reaction gave more pronounced results than that giving this reaction. The fall of the heart beat and respiration was more rapid, the fall lower, and the recovery slower. Since in this solution no proteid was present, all having been converted into simple chemical structures as aromatic and amino compounds, we must look among these for the main cause or causes of the marked fall in blood-pressure. As in the preceding experiments the same results were obtained with one or both vagi sectioned.

We have thus in the products of the decomposed pancreas substances that experimentally at least are capable of producing a

marked fall in blood-pressure. This substance or these substances are present in the gland shortly after necrosis begins, in combination with the trypsin, the peptones, and albumoses. The biuret reaction tends to disappear after the lapse of a short time, and there is left the aromatic and amino compounds.

In addition to the experiments on dogs, thirty white rats were inoculated intraperitoneally with pancreatic material in various degrees of decomposition, with the result that twenty-one died, nine recovered; but those that recovered showed marked evidence of severe effects for a day or two after inoculation. The material not giving the biuret reaction seemed especially toxic. Seven died within twelve hours after inoculation with this solution. However it appeared that when the material had stood for three or four weeks the toxicity was not as great and recovery was more likely.

Bacteriological inoculations were made from the peritoneal cavities of all the animals dying, and from only one was a pathogenic germ isolated, namely Micrococcus pyogenes aureus. The blood-vessels of the peritoneum of most of the rats were injected, but no exudate was present.

CONCLUSIONS.

From these results it is permissible to draw the following conclusions which may be used to explain certain phenomena associated especially with the onset of acute pancreatitis.

1. The sudden marked collapse of acute pancreatitis has as its most important cause a toxin or toxins derived from broken down pancreatic tissue. This toxin or toxins seem to be most powerful in the stage of degradation just after the disappearance of the biuret reaction and are probably of the nature of aromatic and amino-compounds.

2. There are various contributing factors of toxic action as peptone and trypsin, but these are to be regarded as of secondary importance.

3. Mechanical irritation as stretching of the peritoneum and irritation of the cæliac plexus is a secondary cause.

4. The glycerine produced through the action of steapsin cannot be regarded as an important cause of collapse.

EXPERIMENTAL PLEURISY—RESOLUTION OF A FIBRINOUS EXUDATE.

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The purpose of the present study has been to determine the part taken by enzymes in the resolution of a fibrinous exudate. Previous studies have shown that those cells, which with inflammation act as phagocytes and are capable of intracellular digestion, contain two proteolytic enzymes. One enzyme first described by Friedrich Müller is peculiar to the polynuclear leucocytes with fine granulation and is most efficient in the presence of a weakly alkaline medium. The second enzyme, which is present in the mononuclear phagocytes or macrophages, digests only in the presence of acid and resembles the so-called autolytic enzyme which is present in almost all tissues of the body. The action of these enzymes is best studied in exudates produced by sterile inflammatory irritants, since bacteria themselves contain proteolytic enzymes which may be mistaken for the enzymes peculiar to the exudate.

When turpentine is injected into the subcutaneous tissue of the dog an abscess containing thick, yellowish white pus is formed and causes extensive solution of tissue and undermining of the skin. The purulent fluid contains cells in great number many of which undergo disintegration so that the small amount of turbid fluid which by centrifugalization can be separated from the cells of the exudate contains fat droplets and other products doubtless derived from destroyed leucocytes; no fibrin is present. I have previously shown[1] that the serum removed by centrifugalization from this purulent exudate, unlike the serum of the blood, fails to inhibit the proteolytic enzyme contained in the polynuclear leucocytes (leucoprotease).

When an equal quantity of turpentine, one cubic centimeter, is injected into the pleural cavity of the dog, the reaction which re-

[1] *Jour. of Exper. Med.*, 1906, viii, 536.

sults is very different. Fluid accumulates in large quantity so that at the end of three days there may be one hundred or more cubic centimeters. During the first two or three days after injection this fluid is coagulable, and fibrin in considerable quantity is deposited upon the pleural surfaces. The accumulation of fluid and its gradual disappearance may be followed with considerable accuracy during life by percussion of the animal's chest. Only by such examination during life has it been possible to follow the course of each experiment and to determine when the inflammatory reaction is progressing and when receding.

Sero-fibrinous Pleurisy.—In all instances turpentine has been injected into the right pleural cavity. When the dog is standing dullness on percussion caused by the heart extends a variable distance to the right of the median line; occasionally there is at the usual level of the uppermost pair of teats absolute dullness or flatness on percussion one or two centimeters from the mid-line, but in most instances there is no absolute dullness, but merely impairment of resonance (relative dullness) over an area corresponding to the underlying heart two or three centimeters to the right of the mid-line. With accumulation of fluid there is increase of dullness in the dependent part of the chest so that the gradual rise of the upper level of dullness measured from the median line may be determined from day to day. Immediately above the level of absolute dullness is an area of impaired resonance usually one or two centimeters across. By changing the position of the animal so that the vertebral column is upright, there is change in the distribution of dullness on percussion corresponding to change in the position of the fluid, but this change at times occurs slowly and is evident only after the upright position has been maintained for several minutes. In the upright position the area of flatness measured to the right from the median line diminishes, but does not return to the normal limit present before injection of turpentine, and at the same time flatness appears in the back over the lowermost part of the chest next to the vertebral column. Movable dullness is recognizable here when the limit of hepatic dullness has been previously mapped out with the animal in its normal position. Aspiration of fluid has been much facilitated by inserting the

needle in this area of movable dullness and withdrawing fluid while the animal is held in upright position.

By carefully determining by percussion the height of fluid from day to day, it has been found that almost constant changes follow the injection of turpentine. The gradual increase of fluid during three days and its subsequent disappearance is illustrated by the following experiment, in which the height of fluid was determined in the normal horizontal position.

Experiment I.

	Absolute Dullness.	Relative Dullness.	
Before injection	0 cm.	2.9 cm.	1 c.c. turpentine injected into right pleural cavity.
After one day	3.1 cm.	4.8 cm.	Animal is inert.
After two days	4.7 cm.	6.0 cm.	Animal appears sick.
After three days	5.5 cm.	7.0 cm.	Animal is somewhat inert.
After four days	4.8 cm.	7.0 cm.	Animal appears to be well.
After five days	0 cm.	6.3 cm.	

The animal has been killed at the end of five days; the right pleural cavity contains 43 c.c. of deep red fluid (containing blood) and over the ventral half of the external surface of the lung is a thin layer of fibrin.

The foregoing experiment serves to indicate the limitations of percussion applied to the dog's chest, for although the cavity has contained forty-three cubic centimeters of fluid there has been no absolute dullness on percussion.

In nine of twelve experiments the maximum height of fluid was present at the end of three days after injection of one cubic centimeter of turpentine into the right pleural cavity. In three instances the maximum amount of dullness was present at the end of two days. Having reached a maximum the dullness disappears with much rapidity. With subsidence of fluid it is noteworthy that the area of impaired resonance often does not diminish as rapidly as the absolute dullness. Examination after death indicates that such impairment of resonance on percussion may be caused by a layer of fibrin still present over the surface of the lung. Moreover, it has been found that even when, at the end of six or more days, fluid has completely disappeared from the cavity resonance near the sternum may still be absent or impaired, because there is in this situation a layer of fibrin of variable thickness. Careful

percussion of the chest during the course of such experiments has the further advantage that the success of attempted intrapleural injection may be determined after one or two days. If the injecting needle has entered the lung a sterile intrapulmonary abscess results and instead of dullness over the dependent part of the chest percussion shows flatness of an especially hard resistant character about the site of injection. By percussion after aspiration it is possible to determine if fluid present in the chest has been almost completely removed.

At the end of two or three days after injection of turpentine, when there is greatest dullness on percussion, the fluid in the pleural cavity may be one hundred cubic centimeters or more. The fluid is usually turbid and pale yellow, but the presence of red corpuscles may give it a reddish color. Cells are present in such small number that after centrifugalization they are found to represent only a very small proportion, usually about half of one per cent. of the total volume of the exudate. At the end of five or six days fluid has in most instances completely or almost completely disappeared from the cavity. The following table shows the quantity of fluid present in the chest at autopsy performed at various intervals after the injection of one cubic centimeter of turpentine.

TABLE I.

Time after Injection	2 Days.	3 Days.	4 Days.	5 Days	6 Days.	7 Days.
	106 cm.	22 cm.	130 cm.	43 cm.	1 cm.	0.5 cm.
	96 cm.		38 cm.	1 cm.	0 cm.	
			25 cm.		0 cm.	
			7 cm.			

These figures show that the fluid present in the pleural cavity varies considerably in different animals at the same stage of the inflammatory reaction although the same irritant has been injected; percussion of a much larger number of animals has shown that the maximum accumulation of fluid is reached at the end of two or more frequently of three days. The rapidity with which fluid disappears is subject to wide variation though in most instances at the the end of five or six days the quantity remaining is trivial.

The intensity of the inflammatory reaction bears no constant relation to quantity of irritant injected. The volume of the fluid

which accumulates in the chest during the first three days after intrapleural injection is roughly indicated by the extent of dullness on percussion, the animals employed being dogs of fairly uniform size weighing four or five kilos. The following experiments are quoted to show the variable effect produced by injection of one half, of one, or of two cubic centimeters of turpentine.

After Injection of One Half Cubic Centimeter of Turpentine.—After injection of 0.5 cubic centimeter of turpentine the maximum amount of dulness was present in one animal after one day, in one after two days, in the third after three days. The least (Experiment II) and the greatest (Experiment III) amount of dullness resulting is recorded as follows:

	Experiment II.		*Experiment III.*	
	Absolute Dullness.	Relative Dullness.	Absolute Dullness.	Relative Dullness.
Before injection	0 cm.	1.1 cm.	0 cm.	2.4 cm.
After one day	0 cm.	4.8 cm.	4.3 cm.	5.4 cm.
After two days	0 cm.	2.6 cm.	5.8 cm.	7.7 cm.
After three days	0 cm.	1.1 cm.	7.6 cm.	9.2 cm.

After Injection of One Cubic Centimeter of Turpentine.—Of ten experiments, in three the maximum amount of dullness was present at the end of two days, in seven at the end of three days. The least (Experiment IV) and the greatest (Experiment V) dullness was as follows:

	Experiment IV.		*Experiment V.*	
	Absolute Dullness.	Relative Dullness.	Absolute Dullness.	Relative Dullness.
Before injection	0 cm.	2.2 cm.	0 cm.	3.9 cm.
After one day	0 cm.	2.2 cm.	4.5 cm.	8.2 cm.
After two days	3.8 cm.	5.2 cm.	7.0 cm.	8.6 cm.
After three days	0 cm.	3.5 cm.	7.7 cm.	9.2 cm.

After Injection of Two Cubic Centimeters of Turpentine.—In four experiments the maximum of dullness occurred after two days, and in two experiments after three days. Experiments showing the least (Experiment VI) and the greatest dullness (Experiment VII) are as follows:

	Experiment VI.		*Experiment VII.*	
	Absolute Dullness.	Relative Dullness.	Absolute Dullness.	Relative Dullness.
Before injection	0 cm.	2.0 cm.	0 cm.	2.6 cm.
After one day	2.9 cm.	5.0 cm.	3.6 cm.	7.0 cm.
After two days	4.5 cm.	6.0 cm.	7.2 cm.	10.6 cm.
After three days	4.4 cm.	5.4 cm.	10.2 cm.	12.4 cm.

The observations just described show that although there is in general an increase in the amount of exudation corresponding to an increase of the irritant injected, there is such wide variation that the greatest exudation of fluid after injection of one half cubic

centimeter of turpentine may exceed the least exudation caused by two cubic centimeters. Peculiarities of the animal or conditions affecting it obviously have a very important part in determining the intensity of the inflammatory reaction.

Fibrin which forms a layer between the surface of the lung and the parietal pleura is deposited in greatest abundance in the most dependent part of the cavity. The pleural surface of the pericardium and the ventral half of the external surface of the lung, particularly near the sternum, is at the end of two or three days covered by a layer of fibrin a millimeter or more in thickness; whereas over the posterior border of the lung and over the adjacent external surface there is little, if any, fibrin. Everywhere the surfaces of the visceral and parietal pleuræ have lost their normal appearance, and are dull. During the early stages of the inflammatory process the fibrin is opaque white and succulent in appearance, but later, at a time when fluid has disappeared from the cavity, that is at the end of five or six days, fibrin forms a much thinner layer and is dry, gray-white and tough. This layer of fibrin which is usually attached very loosely to the pleura gradually diminishes and at the end of about two weeks or less has completely disappeared. The pleural cavity has become entirely normal or a few organized fibrous shreds represent the only evidence of former inflammation.

Microscopical examination of the fibrin present at the end of two days shows that it is fairly dense in structure, consisting of irregular strands or lamellæ containing imbedded in their substance cells with a single oval or somewhat irregular nucleus and abundant protoplasm. Polynuclear leucocytes are present in considerable number; within the substance of the fibrin they are less numerous than the mononuclear cells but occur in collections of considerable size in spaces between the strands. In scattered areas, on the contrary, the fibrin itself may be infiltrated with polynuclear leucocytes in considerable number. Where fibrin rests upon a serous surface endothelial cells have completely disappeared.

At the end of two days the polynuclear leucocytes which are present are well preserved, the nucleus having the typical multilobed form, but after four or five days both polynuclear and mononuclear cells have in large part disappeared from the fibrin which in places

may be nearly free from cells. Polynuclear leucocytes which remain show various forms of degeneration; the nucleus stains deeply and may be broken into a large number of small round fragments, or may have completely disappeared leaving a necrotic cell recognizable by its size and by comparison with neighboring cells, which have undergone less advanced change. Though the mononuclear cells which at an earlier period have been embedded within the strands of fibrin have in many places disappeared, similar cells are numerous where fibrin is in contact with the underlying pleura. In places occur foci of large mononuclear cells, massed together and situated between strands of fibrin where polynuclear leucocytes were previously most abundant.

At the end of six days organization of fibrin is beginning and blood vessels accompanied by lymphoid and large mononuclear cells in variable number are making their way from the pleural tissue which has often undergone considerable proliferation into the fibrin which in corresponding degree is disappearing. The relation of one or other type of cell to the solution and disappearance of fibrin is not discoverable in sections of the tissue, though not infrequently those large mononuclear cells with abundant protoplasm which act as phagocytes (macrophages) are abundant where fibrin is obviously undergoing solution.

Pleurisy on the Left Side Caused by Injection into the Right Pleural Cavity.—The left pleural cavity in most instances remains unaffected by the inflammatory process occurring in the right, but occasionally there is accumulation of fluid containing a small number of leucocytes, and fibrin is deposited especially upon the pleural surface of the mediastinum and of the pericardium and upon the adjacent surface of the left lung. This inflammatory reaction is doubtless referable to irritation of the thin membrane which forms a considerable part of the mediastinum, separating the right and left pleural cavities. In several instances the left pleural cavity has been found to contain a considerable quantity of fluid at a time when fluid has almost completely disappeared from the right cavity which received the irritant. In one instance at the end of four days the right cavity contained only seven cubic centimeters of fluid, whereas the left cavity contained seventy-five cubic centimeters;

in a second experiment at the end of four days the right cavity contained ten cubic centimeters and the left, one hundred and fifty cubic centimeters of fluid; the reaction was at its height in the left pleural cavity at a time when it was subsiding in that cavity which had received the injection.

The sero-fibrinous pleurisy which has been described presents an opportunity to study the accumulation and disappearance of fluid and, as well, the formation and solution of a fibrinous exudate. The whole process of resolution may be studied under conditions uncomplicated by the presence of a living multiplying microorganism which may contain enzymes and other bodies similar to those present in the cells of the inflammatory exudate. Moreover the sero-fibrinous inflammation which is caused by turpentine does not differ in any essential features from similar pleurisies which in man are caused by Diplococcus lanceolatus and other microorganisms, the changes which occur reproducing with considerable accuracy the human lesions. The analogy of this type of pleurisy to acute lobar pneumonia is close and resolution in the two processes is in its underlying features doubtless the same.

Fate of the Irritant.—In order to determine the relation of the reaction to the irritant the fate of the injected turpentine is of much importance. During the first three days at a time when the fluid is increasing, turpentine can be recognized by its characteristic odor in fluid removed from the chest. At a later period it is no longer recognizable, although in the purulent exudate removed from an abscess caused by subcutaneous injection of turpentine, the substance is recognizable by its odor at a much later period. Accumulation of pleural exudate ceases perhaps when the irritant has been destroyed or removed from the cavity, and subsequently absorption proceeds with variable rapidity, being completed within from four to six days after injection of turpentine.

Coagulability of the Exuded Fluid.—When aleuronat in suspension is injected into the pleural cavity of the dog serum containing leucocytes accumulates in large quantity, but fibrin is not deposited upon the pleural surfaces; samples of fluid removed from the chest fail to undergo noteworthy coagulation, and after stand-

ing for a considerable time only a few shreds form about the cells which have subsided. When, on the contrary, turpentine has been used to excite inflammation, fluid removed from the pleural cavity during the first three days after injection of the irritant undergoes such firm coagulation within a few minutes that a small tube containing it may be inverted. The coagulum occupies the entire volume of the fluid. At the end of two days or occasionally after three days coagulation is less complete and the coagulum which forms perhaps after the fluid has stood for a considerable time, occupies only a small part of the entire volume of fluid, whereas in some instances, only a small shred of fibrin may be formed. Fluid removed at the end of five or six days fails to undergo coagulation.

Such casual examination of specimens of fluid removed at various intervals after injection of the irritant suggests that the degree of coagulation is dependent upon the amount of fibrinogen in the fluid and bears no relation to the presence or absence of fibrin ferment. After the third day, at a time when little if any fresh serum finds its way from the blood vessels into the cavity, fibrinogen has been, doubtless, almost completely or completely deposited to form the mass of fibrinous exudate adherent to the pleural surfaces. It is not impossible that this layer of fibrinous deposit is derived from a greater volume of fluid than is present in the cavity at any one time, the serum of the blood forming by weight only from 0.2 to 0.4 per cent. of fibrin. During the period when fluid is accumulating in the cavity it is probable that it is at the same time escaping, in part at least, by way of the lymphatics. Fluid is entering in greater quantity than it is leaving the cavity and that which enters is adding to the fibrinous deposit. After the third day, however, little fluid is entering, while much is absorbed and hence the supply of fibrinogen has ceased.

Enzymes of the Exuded Fibrin.—Previous studies[2] have shown that the cells of inflammatory exudates contain two proteolytic enzymes, demonstrable by subjecting coagulable proteid to their action. Proteolysis caused by these enzymes has been measured by estimating by means of the Kjeldahl method the amount of nitrogen of coagulable proteid (heated blood serum) converted by digestion at

[2] *Jour. of Exper. Med.*, 1905, vii, 316; *ibid.*, 1906, viii, 410.

body temperature into incoagulable form. The activity of enzymes contained in the fibrinous exudate may be tested by a more direct and simple method; disintegration and solution of the exuded fibrin may serve as an index of the activity of the proteolytic enzymes which it contains.

Particles of exuded fibrin, freed from serum by washing in normal salt solution and pressing one or more times between pads of sterile gauze, undergo autolysis when suspended in salt solution (0.85 per cent.). The necessity of removing the serum of the exudate is demonstrated by the following experiment in which measured quantities of serum have inhibited the self-digestion of particles of fibrin of approximately equal size suspended in fluid of which the volume has been brought to twenty cubic centimeters by addition of normal salt solution. Putrefaction is prevented by addition of toluol. The occurrence of digestion as far as it is indicated by disintegration and solution of the particle of fibrin has been indicated by the plus sign (+) and the degree of disintegration by one or more such signs dependent upon the appearance of the fibrin still undissolved.

Experiment VIII.

Fibrin of Exudate.	Degree of Digestion after 3 Days at 37° C.
With no serum of exudate	+ + +
" 0.5 c.c. serum of exudate	+ +
" 1.0 " " "	+
" 2.0 " " "	0
" 5.0 " " "	0

Under the conditions of the experiment two cubic centimeters of the serum are sufficient to preserve the exuded fibrin unchanged during three days at 37° C., while smaller quantities appreciably inhibit its self-digestion.

The cells of the serous inflammatory exudate produced by injection of aleuronat into the pleural cavity contain two enzymes, one of which, leucoprotease, acts in the presence of an alkaline medium, whereas the other, which for convenience may be designated lymphoprotease, acts only in the presence of acid; lymphoprotease is present in greatest quantity in the exuded cells during the later stages of inflammation. In view of these facts fibrin of the exu-

date present in the pleural cavity at various intervals after injection of turpentine has been subjected to the action of weak acetic acid and of dilute solutions of sodium carbonate. The layer of fibrinous exudate stripped from the surface of the lung is freed from serum and cut into particles of approximately equal size. Each particle of fibrin is suspended in five cubic centimeters of fluid. The test-tube containing fluid and fibrin is tightly closed with a rubber stopper after a small quantity of toluol (five drops) has been added to prevent growth of bacteria. Since the serum of the blood inhibits the action of the enzymes contained in the exudate, one of the equivalent particles of fibrin is for control suspended in five cubic centimeters of fluid containing one cubic centimeter of blood serum; since no digestion occurs and the particle of fibrin remains unchanged, comparison will show the amount of solution in the other tubes. All of the tubes have been incubated at 38° C. during from four to seven days.

In the presence of a weak alkaline solution, fibrin may quickly swell and undergo conversion into a viscid, semi-fluid substance, which is, in part, dissolved in the overlying fluid. Weak acid causes the fibrin to contract and break up into a fine, powder-like sediment. In either case, when digestion occurs, the fluid becomes turbid, while in absence of digestion, the fluid about the unchanged fibrin remains almost clear. Slight digestion may be indicated by erosion of the fibrin recognizable only by comparison with the control suspended in diluted blood serum.

Further evidence of proteolytic digestion may be obtained by testing for incoagulable digestion products the fluid overlying what remains of the fibrin. The simple method of testing for peptones suggested by Obermeyer[3] and employed by Starling,[4] Halliburton and Colls,[5] and others has been used, the tube containing blood serum again serving as control. Three cubic centimeters of the fluid overlying the remains of the fibrin are mixed with an equal quantity of a ten per cent. solution of trichloracetic acid. After the mixture has been shaken and allowed to stand for a short time, the precipitate

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[3] *Med. Jahrbücher*, 1888, iii, 375. *Ref. Maly's Jahresbericht*, 1889, xix, 7.
[4] *Jour. of Physiol.*, 1893, xiv, 131.
[5] *Jour. of Path. and Bact.*, 1896, iii, 295.

of coagulable proteid is removed by filtration. The filtrate is now tested for biuret reaction. The intensity of the reaction, which has been used by Starling for quantitative estimation, gives indication of the relative amount of peptone and albumose present. Fluid from the control tube uniformly fails to give the reaction, though it contains the native albumins of the serum, whereas, when digestion has occurred, an intense reaction is present.

The following experiment illustrates the character of the digestion which occurs in different media and the method of recording the changes.

Experiment IX.

Exuded Fibrin was Allowed to Digest During 7 Days at 37° C.	Fibrin.		Biuret Reaction.	
	Character of Change	Relative Amount of Solution.	Character of Reaction.	Relative Intensity of Reaction.
With 1 % acetic acid.	Swollen and gelatinous.	?	Negative.	o
With 0.5 % acetic acid.	Dissolved save small amount of sed.	++++	Deep pink.	++++
With 0.2 % acetic acid.	Dissolved save very small amount of sed.	++++	Moderately deep pink.	++++
With neutral reaction.	Slightly eroded.	+	Trace of pink.	+
With 0.2 % sod. carb.	Slightly eroded.	+	Pale pink.	+
With 0.5 % sod. carb.	Slightly eroded.	+	Pale pink.	+
With 1 % sod. carb.	Translucent but intact.	o	Negative.	o
With 1 c.c. blood serum.	Unchanged.	o	Negative.	o

A close parallel exists between the degree of digestion recognizable by naked eye examination of the particle of fibrin and that indicated by the biuret action after precipitation of coagulable proteid by trichloracetic acid. The method employed serves to distinguish simple solution from digestion. It is well known that fibrin of the blood is dissolved by certain salts and by acids; Fermi[6] found that 0.5 hydrochloric acid dissolved but did not peptonize fibrin. The foregoing experiment shows that the exuded fibrin contains an enzyme which dissolves fibrin and converts it into peptone in the presence of weak acetic acid. In stronger acetic acid (one per cent.) the character of the change is wholly different; the fibrin being swollen and broken into almost transparent particles which nearly fill the entire volume of fluid, it is impossible to determine how much solution has occurred. That acid of this strength is

[6] *Zeit. für Biol.*, 1891, xxxviii, 229.

unfavorable for the action of the enzyme contained in the exuded fibrin is shown by the absence of reaction for peptone. With weaker acid, favorable to the action of the enzyme, absolute parallel between macroscopic solution and peptone reaction cannot be expected, for proteid may be converted by continued action of enzyme into decomposition products which do not give a biuret reaction. The following table shows the behavior of fibrin removed from the chest at intervals of from two to ten days after injection of turpentine, when suspended in media differing in reaction:

TABEL II.

Experiment.	No. of Days After Injection.	With 2 per cent. Acetic Acid.		With Neutral Reaction.		With 2 per cent. Sodium Carbonate.	
		Solution of Fibrin.	Biuret Reaction.	Solution of Fibrin.	Biuret Reaction.	Solution of Fibrin.	Biuret Reaction.
A	2	++++	+++	++	+	+	+++
B	2	+++		+		++	
C	3	++++	++++	+	+	+	+
D	4	++++		o		++++	
E	5	++++	++++	+	+	+(?)	+(?)
F	6	++++	+++	o	o	o	o
G	6	+++	++	o	o	o	o
H	6	++++	++++	o	o	o	o
I	9	++++	++++	+++	+	o	o
J	10	+++	+++	o	o	o	o

The table shows that digestion is much less active in a neutral medium than in acid or in alkali. During the first four or five days digestion occurs both in the alkaline and in the acid medium, but at the end of five days the power to digest in the presence of alkali has been lost, so that subsequently little, if any, digestion occurs, save in the presence of acid. It is noteworthy that the ability to undergo digestion in alkali disappears at a time when fluid has undergone almost complete absorption. This relation is not constant, and Experiment G is an exception, the pleural cavity containing sixty-eight cubic centimeters of fluid, in large part encapsulated.

Injection of Leucocytes into the Inflamed Pleural Cavity.—In two experiments which follow the ability of exuded fibrin to undergo self-digestion in the presence of alkali has been increased by injecting into the inflamed chest polynuclear leucocytes in considerable quantity.

Experiment X.—Sterile pus, obtained four days after injection of turpentine into the subcutaneous tissue of the dog has been injected into the pleural cavity two days after the onset of inflammation.

	Absolute Dullness.	Relative Dullness.	
Before injection	0 cm.	3.2 cm.	1 c.c. of turpentine injected into the right pleural cavity.
After 1 day	7.7 cm.	12.5 cm.	Animal is sick.
After 2 days	12.2 cm.	13.9 cm.	14 c.c. pus injected into the right pleural cavity.
After 3 days	16.2 cm.	17.4 cm.	Animal is sick.
After 4 days	13.2 cm.	15.8 cm.	Animal is sick.
After 5 days	11.6 cm.	14.8 cm.	Animal is sick.

The animal has been killed by hæmorrhage under ether. The right pleural cavity contains 175 c.c. of blood-stained fluid. Fibrin is present in large quantity and forms a layer often two millimeters in thickness. In places, this fibrin is grayish, tough and firm, but elsewhere it has a yellowish-white color, and is soft and ragged, as if undergoing disintegration.

Fibrin freed from serum was subjected to autolysis under the conditions previously described.

	Solution of Fibrin.	Biuret Reaction.
With 0.2 per cent. acetic acid	+ + +	+ + +
With neutral reaction	0	+
With 0.2 per cent. sodium carbonate	+ +	+ + +
With 1 c.c. blood serum	0	0

As a result of the injection of fluid containing polynuclear leucocytes in immense number fibrin, removed from the chest five days after the onset of inflammation, digests itself almost as readily in alkali as in acid. Comparison with Table II shows that power to digest in alkali has been augmented. It is, however, not improbable that the pus which was injected two days after the onset of pleurisy contained some turpentine, and, therefore, tended to prolong the effect of the turpentine originally brought in contact with the pleura. To avoid this possibility leucocytes from a purulent exudate were washed before injection.

Experiment XI.—Cells from a sterile, purulent exudate obtained by subcutaneous injection of turpentine were separated from the fluid of the pus and washed by centrifugalization in 0.85 per cent. salt solution; after removal of the supernatant fluid, the cells were injected into the inflamed pleural cavity, three days after the onset of pleurisy.

	Absolute Dullness.	Relative Dullness.	
Before injection	0 cm.	3.9 cm.	1 c.c. of turpentine was injected into the right pleural cavity.
After 1 day	4.5 cm.	8.2 cm.	Animal sick.
After 2 days	7.0 cm.	8.6 cm.	Animal sick.
After 3 days	7.7 cm.	9.2 cm.	14 c.c. washed leucocytes were injected into the pleural cavity.
After 4 days	0 cm.	3.5 cm.	

When the animal was killed by hæmorrhage under ether, the right pleural cavity contained 45 c.c. of deep red serous fluid. Fibrin formed a thick layer which was soft, yellowish-white and succulent. It is not improbable that a part of the injected turpentine entered the substance of the lung, for a small tear was found at the site of injection. Autolysis of fibrin occurred as follows:

	Solution of Fibrin.	Biuret Reaction.
With 0.2 per cent. acetic acid	+ + +	+ + +
With neutral reaction	o	+
With 0.2 per cent. sodium carbonate	+ + +	+ + +
With 1 c.c. blood serum	+ (?)	+

It is noteworthy that the injection of washed leucocytes was followed by no increase of fluid indicated by percussion, as in the preceding experiment, and very marked diminution of dullness was found on the following day. The relative ability of fibrin to digest in the presence of alkali is greater than that indicated by any of the experiments recorded in Table II. Leucoprotease is, indeed, so abundant that one cubic centimeter of blood serum has failed to inhibit its action. Microscopical examination of the soft fibrin which is apparently undergoing disintegration shows that it is infiltrated with polynuclear leucocytes in immense number and suggests the probability that these are in large part leucocytes which have been injected into the cavity, since fibrin at a corresponding interval, after simple injection of turpentine, contains relatively few polynuclear cells.

Anti-Enzymotic Action of the Exuded Fluid.—Since two stages of enzymotic activity are recognizable during the course of the inflammatory reaction, one requiring the presence of alkali, and the other of acid, it is necessary to determine if conditions present in the fluid of the exudate are such that during life these enzymes are brought into action. The serum of the blood and of the exudate furnish the necessary alkalinity for that enzyme which digests in the presence of alkali, and is characteristic of the polynuclear leucocytes. This enzyme is, however, inhibited by the anti-enzymotic activity of the exuded serum. At no time during the course of the fibrinous inflammation caused by turpentine is this anti-enzymotic action of the fluid surrounding the fibrin lost, and, indeed, only when the corresponding enzyme has disappeared from the fibrin has absorption of fluid removed the influence of the anti-enzyme.

The following experiments show that there is no noteworthy diminution of the anti-enzymotic activity of the exuded fluid when tested with leucoprotease, the enzyme of the polynuclear leucocytes. In each experiment twenty milligrams of dry powder, prepared from polynuclear leucocytes, was allowed to act upon heated blood serum during five days at 37° C., and the amount of digestion was determined by the Kjeldahl method;[7] the ability of the exuded fluid to inhibit the activity of the enzyme was determined by comparing this digestion with that which occurred when the same amount of enzyme acted upon the same amount of coagulated proteid in the presence of a half cubic centimeter of the fluid to be tested. These determinations have been made with the assistance of Miss Bertha I. Barker. By "age of the exudate" should be understood the number of days between injection of turpentine and withdrawal of fluid from the chest either by aspiration or after death. The approximate degree of inhibition has been estimated by subtracting the figure (in cubic centimeters of $\frac{1}{10}$ N. sulphuric acid) representing digestion in the presence of serum from that representing unrestrained digestion.

TABLE III.

Experiment.	Age of Exudate in Days.	Control.	Digestion with 20 mgr. Leucoprotease.	Digestion with 20 mgr. Leucoprotease and 0.5 c.c. Serum.	Degree of Inhibition.
A	1	2.4 c.c.	16.4 c.c.	5.45 c.c.	10.95 c.c.
	3	2.4 c.c.	16.4 c.c.	6.1 c.c.	10.3 c.c.
B	3	2.1 c.c.	18.9 c.c.	8.85 c.c.	10.05 c.c.
C	5	2.0 c.c.	18.85 c.c.	7.05 c.c.	11.8 c.c.

The following experiment is scarcely necessary to show that fibrin which has been found to digest only in the presence of acid and hence contains only lymphoprotease fails to undergo autolysis in the presence of the exuded serum. The fibrin and serum were obtained from a dog (Table II., Experiment E) five days after injection of turpentine. Table II. shows that this fibrin underwent almost no autolysis in the presence of alkali. The volume of fluid was brought to five cubic centimeters by addition of normal salt solution.

[7] *Jour. of Exper. Med.*, 1906, viii, 410.

Experiment XII.

	Solution of Fibrin.	Biuret Reaction.
With 0.5 c.c. of exudate serum	0	0
With 1 c.c. of exudate serum	0	0
With 2 c.c. of exudate serum	0	0

The foregoing study shows that the fluid exuded into the chest as a result of the presence of turpentine is coagulable during the first two days of the inflammatory reaction and deposits upon the pleural surfaces a layer of fibrin containing entangled in its substance an immense number of leucocytes. These cells undergo disintegration and set free an enzyme which is capable, in the presence of an alkalinity equal to that of the serum, of dissolving fibrin and other proteid. The serum of the exudate contains an anti-enzyme which in part holds in check this enzyme and is doubtless efficient in limiting its action, thus perhaps protecting injured tissues from destruction. It is difficult to prove that these enzymes are capable of attacking and destroying living tissue, but no facts that are known exclude this possibility. Where polynuclear leucocytes are massed together in the meshes of the compact fibrinous network which forms the layer overlying the lung, enzyme is, perhaps, unrestrained by anti-body, and partial digestion of fibrin results. That such digestion actually occurs is indicated by the appearance of peptone which, absent at the beginning of the process, is present in greatest quantity when, after three days, the inflammatory reaction has reached a maximum.

The Occurrence of Products of Proteolytic Digestion in the Exuded Fluid.—Products of proteolysis have been found in fluid withdrawn at intervals after injection of turpentine. By precipitating the albumin and globulin contained the exuded serum with trichloracetic acid as recommended by Starling, no biuret reaction is obtained with the filtrate at the end of the first day. At the end of the second day no reaction, or only a trace of reaction occurs, but at the end of the third day, when the quantity of fluid in the chest is greatest, a well marked reaction is usually obtained. At the end of four and of five days a reaction is usually obtainable, but it is less intense than after three days. The following table shows the result of tests made at various intervals after injection of turpentine:

TABLE IV.

Reaction for Peptone and Albumoses.

Experiment.	1 Day.	2 Days.	3 Days.	4 Days.	5 Days.
A	0	*T*	++	+	
B				0	
C	0		+++		+
D		0			
E	0	(?)	0	0	
F				+	

The absence of reaction for peptone is indicated by zero (0). The occurrence of a biuret reaction after coagulation of proteids other than peptone is indicated by the signs of addition, the intensity of the reaction in a given experiment being indicated by from one to three such signs. A trace of peptone causing a scarcely perceptible biuret reaction is indicated by (*T*).

Since peptone is absorbed with much rapidity from the chest, the presence of a well marked reaction at the end of three days indicates that formation of proteolytic digestion products has reached a maximum and now exceeds their absorption.

The following experiment was undertaken with the purpose of determining what influence products of digestion by leucoprotease exert upon the progress of inflammation caused by turpentine. It shows with what rapidity such products are absorbed from the inflamed chest.

Experiment XIII.—Products of digestion were obtained by allowing 50 mgr. of leucoprotease, with addition of toluol, to act during four weeks at 37° C. upon 25 c.c. of a mixture of dog's blood serum and normal salt solution, previously heated to 75° C.; 10 c.c. of this fluid sterilized and freed from toluol by heating to 65° C. during one hour were twice injected into the right pleural cavity. This fluid diluted 1:10 (*i. e.*, a dilution probably greater than that produced by the fluid in the chest) gave an intense reaction for peptone.

	Absolute Dullness.	
Before injection	2.3 cm.	1 c.c. turpentine injected into the right pleural cavity.
After 1 day	4.0 cm.	10 c.c. fluid containing digestion products injected. One half hour later fluid withdrawn gave well marked reaction for peptone.
After 2 days	7.8 cm.	Only faint reaction for peptone obtained. 10 c.c. fluid with digestion products again injected.
After 3 days	11.4 cm.	No reaction for peptone.
After 4 days	7.5 cm.	Very faint reaction for peptone.
After 7 days	7.3 cm.	No reaction for peptone.

After the animal had been killed, the pleural cavity was found to contain 25 c.c. of reddish serous fluid, together with an unusually large quantity of fibrin.

Alkalinity of the Inflammatory Exudate.—Since an alkaline reaction is essential for the action of one enzyme of the exudate, and an acid reaction for the other, the reaction of the pleural exudate has been studied. At no time does the alkalinity of the exuded fluid disappear, but throughout the course of the inflammation it is less than that of the blood serum. The alkalinity of the exudate was tested by the method proposed by Engel[8] and modified by Gamble.[9] One cubic centimeter of the exudate to be tested was withdrawn with antiseptic precautions from the chest during life, or was obtained at autopsy immediately after death. The exudate was diluted with one hundred times its volume of distilled water and neutralized with $\frac{1}{100}$ N. sulphuric acid, lacmoid paper being used as indicator. Acid was added until a distinctly acid reaction was obtained, and since acid inadvertently added in excess cannot be accurately neutralized, duplicate determinations were made. With the first specimens alkalinity was determined with a fair degree of accuracy, though usually a slight excess of acid was added. In testing the second specimen, it was possible to use great care in adding acid when the quantity indicated by the first test was approached. Hence the second determination, which has been the smaller, is the more accurate, and is given in the following table, which shows the alkalinity of the exudate determined from two to five days after injection of turpentine. The alkalinity of the blood, which was in a number of instances tested simultaneously, varied for one cubic centimeter from 9.3 to 11 c.c. $\frac{1}{100}$ N. acid.

TABLE V.

Alkalinity of Exudate in Cubic Centimeters of 1–100 N. Sulphuric Acid.

Experiment.	1 Day.	2 Days.	3 Days.	4 Days.	5 Days.
A	—	5.4	—	4.95	—
B	—	—	5.5	—	—
C	—	—	—	—	4.2

At the end of three days after the onset of inflammation, polynuclear leucocytes cease to migrate from the blood vessels into the pleura, and those which are enmeshed in the fibrin undergo necrosis

[8] *Berl. klin. Woch.* 1898, xxxv, 308.

[9] *Jour. of Path. and Bact.*, 1906, xi, 124.

and disappear. At the end of five or six days, fibrin no longer contains that enzyme which is peculiar to the polynuclear leucocytes; fibrin, therefore, fails to disintegrate in the presence of weak alkali, but readily dissolves in acid. Though partial solution of fibrin doubtless referable to the enzyme of the polynuclear leucocytes, has occurred within the chest during the first five days, resolution is not complete, and at the end of the first week of inflammation a thin layer of fibrin still covers the pleural surface of the pericardium and anterior surface of the lung near the median line, and in less quantity is present between the base of the lung and the diaphragm.

Effect of Carbon-Dioxide on the Enzyme Present in Exuded Fibrin.—Evidence which has already been discussed shows that the fibrin which remains unresolved by leucoprotease contains an enzyme which acts only in the presence of weak acid; nevertheless fluid in the chest, which is much less alkaline than the blood and diminishes slightly in alkalinity with the progress of inflammation, at no time approaches a neutral or acid reaction. At a later period, when fluid has disappeared from the chest, fibrin is still moistened by lymph which has an alkaline reaction, and though the fibrin contains a proteolytic enzyme capable of digesting in an acid medium, conditions favorable for the action of such an enzyme are at first sight wholly wanting. Nevertheless, the possibility has suggested itself that carbon-dioxide may play the part of an acid, and in the body afford conditions favorable to the slow solution of fibrin.

The following experiments have shown that carbon-dioxide may replace the acetic acid previously used to bring in action the enzyme which alone is present during the second stage of the inflammatory reaction caused by turpentine.

Experiment XIV.—Fibrin was obtained from an animal which had received two days before one cubic centimeter of turpentine into the pleural cavity. This fibrin underwent auto-digestion in the presence both of acid and of alkali, but was only slightly altered when suspended in physiological salt solution. Particles of fibrin were suspended in normal salt solution and through the fluid carbon-dioxide was passed during one half hour. The test-tube containing the fluid was still filled with carbon-dioxide when tightly closed with a rubber stopper after addition of a few (five) drops of toluol. The volume of fluid in each tube was five cubic centimeters, normal salt solution being added to make this volume.

	Digestion of Fibrin.	Biuret. Reaction.
With 0.2 per cent. acetic acid (5 days at 37° C.)	+ + + +	
With salt solution alone (5 days at 37° C.)	+	
With 0.2 per cent. sodium carbonate (5 days at 37° C.)	+ + +	
With 1 c.c. blood serum (5 days at 37° C.)	0	
With salt solution alone (2 days at 37° C.)	0	+
With salt solution and carbon-dioxide (2 days at 37° C.)	+	+ +
With salt solution alone (4 days at 37° C.)	+	
With salt solution and carbon-dioxide (4 days at 37° C.)	+ + +	

In the following experiment fibrin which digested only in the presence of acid was used. The experiment repeats and confirms Experiment XIV and demonstrates, moreover, that carbon-dioxide brings this enzyme into action, even though blood serum (one cubic centimeter) is present.

Experiment XV.—The fibrin employed was obtained from the pleural cavity of a dog, nine days after the injection of turpentine. Digestion occurred during seven days at 37° C.

	Digestion of Fibrin.	Biuret Reaction.
With 0.2 per cent. acetic acid	++++	++++
With 0.2 per cent. sodium carbonate	0	0
With normal salt solution	+	0
With salt solution and carbon-dioxide	+++	++
With salt solution and 1 c.c. blood serum	0	0
With salt solution, 1 c.c. blood serum and carbon-dioxide	++++	++

The following experiment confirms those which have just been described, and again shows that in the presence of a small quantity of blood serum (half of a cubic centimeter) active proteolysis occurs when fibrin which digests only with acid is exposed to the action of carbon-dioxide.

Experiment XVI.—Fibrin was obtained from the pleural cavity of a dog ten days after the injection of turpentine.

	Digestion of Fibrin.	Biuret Reaction.
With 0.2 per cent. acetic acid (5 days at 37° C.)	+++	++++
With 0.2 per cent. sodium carbonate (5 days at 37° C.)	0	0
With salt solution (5 days at 37° C.)	0	0
With 1 c.c. blood serum (5 days at 37° C.)	0	0
With 0.5 c.c. blood serum (2 days at 37° C.)	0	0
With 0.5 c.c. blood serum and carbon-dioxide (2 days at 37° C.)	+	++
With 0.5 c.c. blood serum (6 days at 37° C.)	++	0
With 0.5 c.c. blood serum and carbon-dioxide (6 days at 37° C.)	++	+++

The foregoing experiments, which show that carbon-dioxide can bring into action the enzyme remaining in a fibrinous exudate after disappearance of leucoprotease, suggest that carbon-dioxide may favor resolution of fibrin remaining in the pleural cavity during the latter stage of pleurisy caused by turpentine and doubtless by other agencies. Some plausibility is given to this hypothesis by the observations of Ewald,[10] who found that the carbon-dioxide tension of an exudate increases with its age. He studied the pleural exudate from fourteen cases of pleurisy and found that the total volume of carbon-dioxide which could be withdrawn from the exudate with Pflüger's pump after the addition of acid varied from 29.54 to 63.84 per cent., the higher figure being obtained when the exudate had been present in the chest during a considerable length of time. In purulent fluid which had collected quickly and contained leucocytes in great abundance, carbon-dioxide was present in much smaller quantity, whereas the smallest figures (7.92 and 8.05 per cent.) were obtained with pus from abscesses.

CONCLUSIONS.

Fibrinous pleurisy produced by a sterile inflammatory irritant offers opportunity for study of the part taken by enzymes of leucocytes in the resolution of a fibrinous exudate. When turpentine is injected into the subcutaneous tissue of the dog, an abscess results, but when an equal quantity of turpentine is injected into the pleural cavity, there is abundant exudation of coagulable fluid and the serous surfaces are covered by a layer of fibrin. Accumulation of fluid which can be followed during life by percussion of the animal's chest reaches a maximum at the end of three days, and then gradually subsides, so that at the end of six days, in most instances, the cavity contains no fluid. Fibrin, though diminished in amount at the time when fluid has been absorbed, is still present, and gradually disappears; at the end of two or three weeks the cavity has returned to the normal, save for a few organized adhesions.

Turpentine injected into the right pleural cavity may cause serofibrinous pleurisy on the left side; this inflammation may reach a

[10] Ref. *Maly's Jahresbericht* 1874, iv, 421.

maximum intensity at a time when pleurisy on the right side is subsiding.

During the early stage of inflammation fibrinous exudate, freed from the serum by washing in salt solution, undergoes digestion when suspended in an alkaline (0.2 per cent. sodium carbonate) or in an acid medium (0.2 per cent. acetic acid). At the end of five days, at a time when fluid is disappearing from the pleural cavity, digestion fails to occur in an alkaline medium, but occurs with much activity in the presence of acid.

During the first stage of the inflammatory reaction, when fluid is abundant and the fibrin which is present digests in alkali, thus indicating the presence of leucoprotease, polynuclear leucocytes are very numerous in the meshes of the fibrin. In the second stage, the exuded fibrin contains only one enzyme digesting in the presence of acid. At this time polynuclear leucocytes have disappeared and only mononuclear cells are embedded in the fibrin.

Products of proteolytic digestion, namely, peptone and albumose, absent in the exuded fluid during the first day or two days of inflammation, are present after three days and are found in less quantity at a later period.

The exuded fluid does not at any stage of the inflammatory reaction lose it spower to inhibit both enzymes contained in the leucocytes.

The exudate remains alkaline throughout the period of inflammation, but its alkalinity is less than that of the blood and diminishes slightly with the progress of inflammation.

Since the acids, which in vitro favor the action of the enzyme, present alone during the second stage of the inflammatory reaction, do not occur in the body, the possibility has suggested itself that carbon-dioxide brings this enzyme into action. If carbon-dioxide is passed through normal salt solution in which strips of such fibrin are suspended, digestion is greatly hastened. The normal inhibition exerted by blood serum upon the enzyme is overcome by carbon-dioxide and in the presence of a small quantity of blood serum, carbon-dioxide causes greater enzymotic activity than in the presence of salt solution alone.

THE TRANSFORMATION OF SERO-FIBRINOUS INTO PURULENT PLEURISY.

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The preceding study has shown that turpentine (one cubic centimeter) injected into the pleural cavity of the dog causes rapid exudation of a large quantity of coagulable fluid. Fibrin containing a considerable proportion of the leucocytes which migrate from the blood vessels as a result of the chemiotactic action of the irritant is deposited upon pleural surfaces and the serous fluid about this fibrin contains only a small number of leucocytes. In the fluid is an antienzyme which holds in check the enzyme contained in the polynuclear leucocytes, so that resolution of fibrin proceeds slowly, and at no time does the exudate assume the appearance of pus. The pleural surfaces undergo no permanent alteration, and at the end of about two weeks, the pleural cavity has in most instances become normal, save for a few organized adhesions.

When the same quantity of the inflammatory irritant is injected into the subcutaneous tissue, it is not diluted by a large quantity of fluid. A considerable volume of fluid, perhaps with the aid of the negative pressure within the thorax, readily collects in the relatively spacious pleural cavity, whereas accumulation of fluid in the subcutaneous tissue is checked by the increase of tension which occurs when the tissue is infiltrated with fluid and leucocytes. Examination of this pus has shown[1] that leucoprotease of the polynuclear leucocytes so far exceeds anti-body contained in the serum of the pus that the entire exudate undergoes autolysis when placed under suitable conditions.

If the essential difference between the suppurative inflammation of the subcutaneous tissue with solution of tissue, on the one hand, and the sero-fibrinous pleurisy, with which for a considerable time fibrin remains undissolved, on the other hand, is the relative

[1] *Jour. of Exper. Med.*, 1906, viii, 536.

proportion of enzyme and antibody, it is probable that sero-fibrinous pleurisy may be converted into empyæma either by increasing the quantity of enzymes or decreasing the quantity of anti-enzyme. The following experiments indicate though they do not conclusively demonstrate that a sero-fibrinous may be converted into a purulent pleurisy by either of the methods which have been mentioned; nevertheless they exhibit very clearly, I believe, the mechanism by which the transition occurs.

If from one half to two cubic centimeters of turpentine are injected into the pleural cavity, turpentine is recognizable by its odor in the pleural exudate until the end of about three days, at a time when accumulation of inflammatory products has reached a maximum. By subsequent injections of the same irritant, there is continued exudation of fluid and emigration of leucocytes. A second injection of turpentine, three days after the first, increases the quantity of exuded fluid and prevents the disappearance of leucoprotease from the fibrin.

Experiment XVII.—One cubic centimeter of turpentine was injected into the right pleural cavity of a dog, and three days later the same quantity was again injected.

	Absolute Dullness.	
Before injection	1.2 cm.	1 c.c. turpentine injected.
After 1 day	2.9 cm.	
After 2 days	5.5 cm.	
After 3 days	7.0 cm.	Animal sick; 1 c.c. turpentine again injected.
After 4 days	14.8 cm.	Animal very sick; dyspnœa.
After 5 days		Animal found dead.

The right pleural cavity contains 165 c.c. deep red serous fluid. Loosely attached to the surface of the lung and to the diaphragm is a layer of soft yellowish-white fibrin measuring 3 or 4 mm. in thickness. At the upper part of the sternum is a walled-off cavity containing 5 c.c. of purulent fluid. The left pleural cavity is normal.

The ability of the fibrin present to undergo autolysis after incubation during seven days in various media is indicated by the following table.

	Solution of Fibrin.	Biuret Reaction.
With 0.2 per cent. acetic acid	+	+ + +
With neutral reaction	+	+
With 0.2 per cent. sodium carbonate	+ + + +	+ + + +
With 1 c.c. serum of exudate	+	+

[a] Biuret reaction was tested with filtrate obtained after precipitation of coagulable proteid by trichloracetic acid (see description of methods in preceding article).

It is noteworthy that power to digest in an alkaline medium indicating the presence of leucoprotease is far greater than that observed in any of the experiments recorded in Table II of the preceding article. Furthermore, one cubic centimeter of the exuded serum fails to inhibit autolysis.

In the following experiment two injections of turpentine have produced a typically purulent exudate at the end of seven days after the first injection.

Experiment XVIII.—The animal received 0.5 c.c. turpentine in the right pleural cavity and three days later an equal quantity.

	Absolute Dullness.	
Before injection	0 cm.	0.5 c.c. turpentine injected.
After 1 day	4.3 cm.	Animal sick.
After 2 days	5.8 cm.	Animal inert.
After 3 days	7.6 cm.	Animal apparently well; 0.5 c.c. turpentine injected over base of right lung.
After 4 days	7.6 cm.	Animal very sick; dyspnœa; area of dullness about site of injection at base of right lung.
After 5 days	9.5 cm.	As before; very marked dyspnœa.
After 7 days	10.7 cm.	As before; killed by hæmorrhage under ether.

The right pleural cavity contained 245 c.c. of fairly thick opaque white purulent fluid. After centrifugalization, cells are found to represent 35 per cent of the volume. Within the cavity is a voluminous mass of ragged, often semisolid, yellowish-white fibrin which is evidently in process of disintegration. The surface of the lung is opaque and greenish-white in color.

The serum obtained by centrifugalization of the pleural exudate gives a well-marked reaction for peptone after coagulation with trichloracetic acid. Agar-agar inoculated with pleural exudate remains sterile.

Digestion of the disintegrating fibrin is indicated as follows:

	Solution of Fibrin.	Biuret Reaction.
With 0.2 per cent. acetic acid	+ + +	+ + +
With neutral reaction	+ + + +	+ + + +
With 0.2 per cent. sodium carbonate	+ + + +	+ + + +
With 0.5 c.c. serum of exudate	+ + +	+ + +
With 1 c.c. serum of exudate	+ +	+ +
With 2 c.c. serum of exudate	+ + + +	+ + + +
With undiluted serum of exudate	+ + + +	+ + + +

The serum of the exudate increases rather than diminishes the power of the disintegrating fibrin to undergo digestion.

In the following experiment, three injections of turpentine have caused an intense purulent inflammation.

Experiment XIX.—A primary injection of 2 c.c. turpentine was followed by two injections of 0.5 c.c. after three and four days respectively.

	Absolute Dullness.	Relative Dullness.	
Before injection	0 cm.	2 cm.	2 c.c. turpentine injected into right pleural cavity.
After 1 day	2.9 cm.	5 cm.	Sick.
After 2 days	4.5 cm.	6 cm.	Sick.
After 3 days	4.4 cm.	5.4 cm.	Apparently well; 0.5 c.c. turpentine injected.
After 4 days	2.7 cm.	3.8 cm.	Apparently well; 0.5 c.c. turpentine injected.
After 6 days		12.4 cm.	Sick; marked dyspnœa.

When the animal was killed at the end of six days, the pleural cavity was found to contain 181 c.c. of opaque creamy-white pus (sterile), which after standing became viscid and on centrifugalization failed to separate into a cellular and non-cellular layer. This accumulation of pus was approximately limited to the anterior two thirds of the pleural cavity, the surface of the abscess cavity being covered in large part by a layer of yellowish-white succulent fibrin. Occupying the remainder of the cavity and situated over the dorsal part of the lung and between lung and diaphragm was a mass of loose fibrin containing in considerable quantity encapsulated serous fluid.

The enzyme content of (*a*) the fibrin in contact with the purulent exudate and (*b*) the œdematous fibrin at the base of the lung were tested separately.

(*a*) *Fibrin in contact with purulent exudate.*

	Solution of Fibrin.	Biuret Reaction.
With 0.2 per cent. acetic acid	+ +	+ + +
With neutral reaction	+ + + +	+ + +
With 0.2 per cent. sodium carbonate	+ +	+ + + +

(*b*) *Œdematous fibrin at base of lung.*

	Solution of Fibrin.	Biuret Reaction.
With 0.2 per cent. acetic acid	+ + +	+ + + +
With neutral reaction	+ + +	+ + +
With 0.2 per cent. sodium carbonate	0	+

The absence of very marked increase of absolute dullness, although a large quantity of fluid has been present within the pleural cavity, is perhaps explained by the fact that fibrinous adhesions have held the lung in contact with the chest wall; at the end of six days, there has been, however, impairment of resonance over a considerable part of the chest wall. These facts make it probable that the second and third injections have been confined to that part of the cavity which has been found at autopsy to be the site of purulent exudate. The succulent yellowish-white fibrin in this part of the cavity has undergone digestion with much greater activity in an alkaline than in an acid medium, whereas the fibrin which contains serous fluid more closely resembles that which is present at a corresponding time after a single injection of turpen-

tine, that is, when resolution is in progress; the latter autolyses with much greater activity in the presence of acid than of alkali.

In the preceding article, it has been shown that a single injection of from one half to two cubic centimeters of turpentine is followed by a sero-fibrinous pleurisy which almost constantly undergoes complete recovery with restoration of the pleural cavity to normal. In no instance among thirteen experiments in which examination has been made from three to seven days after injection, and among four experiments of longer duration, has generalized suppuration occurred within the pleural cavity as the result of a single injection of turpentine, though in two instances small macroscopic collections of leucocytes have been found below the pulmonary or parietal pleura. Since these two experiments show that foci of suppuration occur though rarely under the conditions which have been mentioned, their description will precede that of similar lesions which were found far more commonly after the removal of fluid.

Experiment XX.

	Absolute Dullness.	
Before injection	0 cm.	1 c.c. turpentine into right pleural cavity.
After 1 day	3.7 cm.	Sick.
After 2 days	5.4 cm.	Sick.
After 3 days	5.9 cm.	Apparently well; killed.

The right pleural cavity contained 22 c.c. deep red serous fluid (sterile). Over the ventro-external surface of the right lung was a layer of white succulent fibrin, 1 to 1.5 mm. in thickness. Upon the surface of the lung within the fibrin were two slightly raised opaque yellow nodules about 3 mm. across. Microscopical examination shows a central mass of polynuclear leucocytes in various stages of degeneration with nuclear fragmentation and necrosis. In a peripheral zone, mononuclear cells with fairly abundant protoplasm and round deeply staining nuclei are numerous.

Experiment XXI.

	Absolute Dullness.	
Before injection	0 cm.	1 c.c. turpentine into right pleural cavity.
After 1 day	3.6 cm.	Inert.
After 2 days	4.7 cm.	Sick.
After 3 days	5.5 cm.	Inert.
After 4 days	4.8 cm.	Apparently well.
After 5 days	0 cm.	Relative dullness still present over considerable area.

The right pleural cavity contained 43 c.c. deep red serous fluid. Over the ventro-external surface of the lung was a layer of firm gray-white fibrin, 0.5 to 1 mm. in thickness. Between two lobes was a very small (about 2 mm. across)

collection of creamy white fluid, and upon the parietal pleura, near the apex of the lung, was a low elevation (about 7 mm. across). Microscopical examination of the latter shows a sharply localized cavity filled with polynuclear leucocytes, many of which have undergone degeneration; a smaller number of large and small mononuclear cells are present.

When, on the contrary, fluid is removed by aspiration at the height of exudation, localized pleural abscess not infrequently results. At this time, the cavity contains abundant fibrin in the meshes of which are polynuclear leucocytes in great number Experiments in vitro have shown that this fibrin contains leucoprotease in such quantity that autolysis quickly occurs when the influence of the exuded serum is removed. It is probable that a similar process occurs when fluid is removed from the pleural cavity. The occurrence of abscess will depend upon the completeness with which fluid is withdrawn. In some instances, the aspirating needle was inserted about three centimeters from the sternum, the animal being held with the body in the normal horizontal position; withdrawal of fluid is, perhaps, more complete in this position. In other instances, the limit of hepatic dullness in the back beside the vertebral column was determined with the animal in its natural position. When the animal was placed with its body erect, fluid subsided to the dependent part and a zone of movable dullness was definable in the back above the level of the diaphragm. The aspirating needle was inserted within this area, three or four centimeters to the right of the vertebral column.

In seven experiments all the fluid obtainable was withdrawn from the inflamed pleural cavity two days after injection of the irritant, and in five instances, localized abscesses resulted. In the two experiments which follow, abscess formation failed to occur.

Experiment XXII.

	Absolute Dullness.	
Before injection	0 cm.	1 c.c. turpentine injected into right pleural cavity.
After 1 day	2.9 cm.	
After 2 days	4.9 cm.	50 c.c. straw colored fluid aspirated.
After aspiration	0 cm.	
After 3 days	0 cm.	

The animal was killed at the end of seven days. The right pleural cavity contained no fluid; the entire surface of the right lung, save the posterior border, was covered by an irregular firmly adherent layer of tough white fibrin, in places one millimeter in thickness. Microscopical examination shows organization of fibrin; the fibrin remaining contains mononuclear cells but no polynuclear leucocytes.

Experiment XXIII.

	Absolute Dullness.	
Before injection	0 cm.	1 c.c. turpentine injected into right pleural cavity.
After 2 days	12.7 cm.	60 c.c. serous fluid aspirated.
After aspiration	5.2 cm.	
After 3 days	5.1 cm.	
After 4 days	5.1 cm.	
After 10 days	3.0 cm.	Animal killed.

The right pleural cavity contained no fluid; abundant tough white fibrin united the anterior aspect of the lung to the chest wall. Microscopical examination shows advanced organization of fibrin containing only cells of mononuclear type.

In three experiments which follow, small abscesses were found after withdrawal of fluid.

Experiment XXIV.

	Absolute Dullness.	
Before aspiration	2.6 cm.	1 c.c. turpentine injected into right pleural cavity.
After 1 day	3.6 cm.	
After 2 days	4.4 cm.	14.5 c.c. of serous fluid withdrawn.
After aspiration	3.3 cm.	
After 4 days	3.3 cm.	Animal killed.

The right pleural cavity contained 1 c.c. of red turbid fluid. There was much fibrin forming a layer over the ventral aspect of the lung and over the adjacent pericardium. Upon the parietal pleura at the posterior part of the cavity was a yellowish elevation which contained purulent fluid. Within an interlobular cleft upon the anterior margin of the lung was a small enclosed cavity containing thick fluid of purulent appearance.

Microscopical section of fibrin taken at random shows degenerating leucocytes forming collections of considerable size. A section of the nodule upon the parietal pleura shows a well defined abscess cavity within the layer of fibrin; polynuclear leucocytes with nuclear degeneration are mingled in great number with both large mononuclear cells with vesicular nucleus and small cells with deeply staining nucleus.

Autolysis of fibrin from the surface of the lung occurred as follows:

	Solution of Fibrin	Biuret Reaction.
With 0.2 per cent. acetic acid	+ + + +	+ + + +
With neutral reaction	0	0
With 0.2 per cent. sodium carbonate	0	0
With 1 c.c. blood serum	0	0

Experiment XXV.

	Absolute Dullness.	
Before inoculation	1.3 cm.	1 c.c. of turpentine injected into right pleural cavity.
After 2 days	12.2 cm.	85 c.c. straw colored serous fluid withdrawn.
After aspiration	3.0 cm.	
After 3 days	1.5 cm.	
After 6 days	1.5 cm.	
After 9 days		Killed with ether and bleeding.

The right pleural cavity contained no fluid. Fibrin was abundant, forming a layer about 1 mm. in thickness over the surface of the ventral half of the external surface of the lung. The right lung was bound in places to the pericardium, diaphragm, and parietal pleura. The left pleural cavity was normal.

Within a layer of fibrin between the two lobes of the lung is a small flattened cavity containing viscid fluid of purulent appearance. Microscopical examination shows a central mass of necrotic material containing polynuclear leucocytes in various stages of degeneration. Surrounding it is a zone of large mononuclear cells, many of which have ingested the altered polynuclear leucocytes. A zone of fibrin separates these cells from a thick capsule of newly formed connective tissue.

Experiment XXVI.

	Absolute Dullness.	
Before inoculation	2.1 cm.	1 c.c. turpentine injected into right pleural cavity.
After 1 day	4.6 cm.	
After 2 days	6.2 cm.	43 c.c. reddish serous fluid removed.
After aspiration	0 cm.	
After 3 days	2 cm.	Sick.
After 4 days	2 cm.	Sick.
After 9 days	3.5 cm.	Apparently well; killed.

The right pleural cavity contained no fluid. The entire upper lobe was bound by a layer of fibrin, from 0.5 to 1.5 mm. in thickness, to the chest wall and pericardium. Within this fibrin was a small flattened cavity, about 1 cm. across and containing thick whitish fluid having the appearance of pus. Corresponding to this focus of softening, fibrin is found by microscopical examination to have undergone solution; polynuclear leucocytes undergoing degeneration are fairly abundant, but similar cells which are necrotic and without nuclei occur in great number.

In the following experiments, aspiration of all the fluid obtainable has been followed by formation of cavities of considerable size containing sero-purulent or purulent fluid, and this localized suppuration has occurred even though the quantity of fluid withdrawn has been relatively small (10 to 15 c.c.). It is not improbable that fluid has been withdrawn from a part of the chest completely or wholly separated by fibrinous adhesion from the remainder of the cavity.

Experiment XXVII.

	Absolute Dullness.	
Before inoculation	0 cm.	1 c.c. turpentine into right pleural cavity.
After 2 days	7.7 cm.	All fluid (serous) obtainable withdrawn.
After aspiration	2.8 cm.	
After 3 days	4.2 cm.	Sick.
After 4 days	2.5 cm.	Killed.

Within the right pleura walled off by fibrin is a cavity containing 10 c.c. of yellowish purulent fluid; fibrin lining the cavity is soft and ragged and evidently

in process of disintegration. Microscopical examination shows disintegrating fibrin infiltrated with polynuclear leucocytes in immense number. The remainder of the pleural cavity contains about 15 c.c. of reddish serous fluid (sterile on attempted culture), together with a layer of compact gray-white fibrin, 1.5 mm. in thickness. Autolysis of this fibrin occurred as follows:

	Solution of Fibrin.	Biuret Reaction.
With 0.2 per cent. acetic acid	+ + +	+ + +
With neutral reaction	+	+ +
With 0.2 per cent. sodium carbonate	0	0
With 1 c.c. blood serum	0	0

Experiment XXVIII.

	Absolute Dullness.	
Before injection	1.1 cm.	1 c.c. turpentine injected into right pleural cavity.
After 1 day	5.2 cm.	Sick.
After 2 days	7.5 cm.	All fluid (serous) obtainable withdrawn.
After aspiration	4.5 cm.	Apparently well.
After 4 days	4.3 cm.	
After 8 days	4.2 cm.	Apparently well; killed.

In contact with the sternum and displacing the upper lobe of the right lung is a localized pleural abscess containing 40 c.c. of thick creamy white pus. Attempted inoculation of agar-agar has given a negative result. The remainder of the pleural cavity is normal.

The foregoing experiments have suggested the probability that a sero-fibrinous pleurisy of greater intensity might be uniformly converted into empyæma by withdrawal of fluid. In view of the variable results obtained after a single injection of various amounts of turpentine, a second injection, performed at a time when the inflammatory reaction produced by the primary injection had reached its height, was believed to offer the best promise of uniformly increasing the intensity of the reaction. Experiments XVIII and XIX have shown that such double injection may result in suppuration. But in a part of the experiments suppuration has failed to occur. In such cases, after aspiration of all of the fluid which could be obtained within twenty-four hours, a serous has become a purulent exudate. This result is especially noteworthy because the withdrawal of fluid removes from the chest the greater part if not all of the inflammatory irritant, namely turpentine, which is recognizable by its odor in the aspirated fluid.

Experiment XXIX.

	Absolute Dullness.	
Before injection	0 cm.	2 c.c. turpentine into right pleural cavity.
After 1 day	2.3 cm.	
After 2 days	4.1 cm.	
After 3 days	5 cm.	0.5 c.c. turpentine injected.
After 4 days	5.6 cm.	38 c.c. turbid serous fluid (sterile) containing by volume 0.5 per cent. of cells.
After aspiration	0 cm.	

The animal was killed twenty hours after aspiration. The right pleural cavity contained a large quantity of sero-purulent fluid in which were 6.5 per cent. of cells. Fibrin was abundant upon the surface of lung, pericardium, and diaphragm. The basal part of the layer of fibrin, which was usually 2 or 3 mm. in thickness, was fairly firm, but the superficial part was soft, opaque, yellow and partially disintegrated.

Autolysis of fibrin occurred as follows:

	Solution of Fibrin.	Biuret Reaction.
With 0.2 per cent. of acetic acid	+ + + +	+ + +
With neutral reaction	+ + + +	+ +
With 0.2 per cent. sodium carbonate	+ + + +	+ + + +

Experiment XXX.

	Absolute Dullness.	
Before injection	0 cm.	1 c.c. turpentine into right pleural cavity.
After 1 day	4.4 cm.	
After 3 days	4.8 cm.	1 c.c. turpentine again injected.
After 4 days	9.5 cm.	Withdrawn by aspiration 112 c.c. red serous coagulable fluid (sterile) containing 0.25 per cent. cells; serum gives faint reaction for peptone.
After aspiration	0 cm.	Large zone of relative dullness on percussion.
After 5 days	7.9 cm.	Withdrawn for examination 9 c.c. creamy white purulent fluid (sterile) containing 22 per cent. of cells; serum gives marked reaction for peptone.
After 6 days	14.2 cm.	Sample of thick purulent fluid withdrawn; cells form 52 per cent.; marked reaction for peptone.
After 11 days	16.6 cm.	Animal moribund.

The right pleural cavity contains 400 c.c. thick cream-white pus containing 44.5 per cent. of cells. The parietal pleura is much thickened and the surface of the lung has a greenish-gray color. Adherent to the upper lobe of the lung, and in places elsewhere, is a layer of altered fibrin, soft, friable, and with the appearance of necrotic material.

Experiment XXXI.

	Absolute Dullness.	
Before injection	0 cm.	0.5 c.c. turpentine injected into right pleural cavity.
After 1 day	4.5 cm.	
After 2 days	9.7 cm.	
After 4 days	8.2 cm.	0.5 c.c. turpentine again injected.

	Absolute Dullness.	
After 5 days	15.6 cm.	Withdrawn by aspiration 100 c.c. reddish serous fluid containing 0.5 per cent. of cells; faint reaction for peptone.
After aspiration		Over an area about 4 cm. across, absolute dullness remained, but had disappeared in the most dependent part of the chest.
After 7 days	4.5 cm.	Impaired resonance throughout right chest. For examination was withdrawn 1 c.c. of yellowish purulent fluid (sterile) containing approximately 50 per cent. of cells.

At the end of eight days, when the animal was killed, the right pleural cavity contained 168 c.c. of purulent fluid (sterile) with 29 per cent. of cells. The right lung was covered by a mass of fibrin about 5 mm. in thickness, yellowish-white, very soft and evidently in process of disintegration. The serum obtained by centrifugalization of the purulent fluid gives a well marked reaction for peptone (+ +).

Autolysis of the disintegrating fibrin occurred after 7 days at 37° C. as follows:

	Solution of Fibrin.	Biuret Reaction.
With 0.2 per cent. acetic acid	+ + +	+ + +
With neutral reaction	+ + +	+ + +
With 0.2 per cent. sodium carbonate	+ + +	+ + + +
With 1 c.c. blood serum	0	0
With 1 c.c. serum of exudate	+ +	+ +
With 5 c.c. serum of exudate	+ + +	+ + +

In Experiments XXIX and XXX, the fluid present in the pleural cavity as the result of two injections of turpentine has been serous in character at the end of four days after the first injection; aspiration of all the fluid obtainable after puncture at the posterior and basal part of the chest, the animal in upright position, has been followed within twenty-four hours by formation of a purulent exudate. The fluid which was previously thin and reddish, containing less than one per cent. of cells has been replaced by thicker, opaque, white fluid containing, in one instance 6.5 per cent., and in the other 22 per cent. of cells. Fibrin, which with sero-fibrinous inflammation is firm, and grayish-white, has become yellowish-white, and undergoing disintegration is soft or even semi-solid. The fluid part of the exudate which at the time of aspiration exhibited a faint reaction for the products of digestion, twenty-four hours later gives a well-marked reaction. At a later period, the purulent characters of the exudate are intensified, cells becoming more abundant. In Experiment XXXI, examination of the pleural con-

tents has not been made until forty-eight hours after aspiration, and at this time, the cavity has contained fairly thick pus. In Experiment XXX, in which purulent fluid has remained in the chest during six days, fibrin has, in large part, undergone solution.

In order to define the essential difference between a fibrinous and a purulent exudate, tests of the autolytic power of the disintegrating fibrin obtained in the preceding experiments are especially significant when compared with similar tests made with fibrin from a sero-fibrinous exudate (Table II of preceding article); autolysis in an alkaline medium occurs in experiments just described with far greater activity.

In Experiment XXXI, one cubic centimeter of blood serum has completely inhibited digestion of fibrin, yet the same quantity of serum from the purulent exudate has failed to prevent action of the enzyme contained in the fibrin, whereas in the presence of undiluted serum of the exudate, digestion has been more active than with one cubic centimeter, the volume of fluid, after addition of normal salt solution when necessary, being in every instance five cubic centimeters.

The foregoing experiments have been described to illustrate the transformation of sero-fibrinous into a purulent exudate, and to indicate the associated underlying changes which distinguish the latter from the former. They are not believed to indicate that aspiration of fluid in cases of sero-fibrinous pleurisy is a dangerous procedure, changing perhaps a relatively mild pleurisy into empyæma; in the last three experiments which have been described, it is probable that death would have occurred as the result of compression of the lungs and perhaps of the heart at an earlier period, had not aspiration been performed. If they offer any suggestion applicable to treatment of pleurisy, it is that complete withdrawal of fluid with acute pleurisy is undesirable.

These experiments bring further evidence that with an inflammatory reaction which has not reached the degree of intensity indicated by suppuration, leucoprotease of the polynuclear leucocytes is held in check by antienzyme of the exuded serum. Since the pleural cavity presents conditions favorable for the accumulation of fluid, the inflammatory irritant is in the first place rapidly di-

luted; after a singly injection of turpentine, coagulable fluid, together with polynuclear leucocytes rapidly accumulate, but at no stage of the reaction does enzyme overcome the anti-enzyme.

Polynuclear leucocytes which are present within the layer of fibrin deposited upon the surface of the lung removed in part from influence of the serum cause partial solution of this fibrin, but within five days after the onset of pleurisy produced by a single injection of the irritant undergo autolysis and disappear. After aspiration of fluid, two processes may occur, in some instances, side by side. Withdrawal of fluid may, it appears, hasten autolysis and disappearance of polynuclear leucocytes, so that fibrin removed from the chest four days after the onset of pleurisy (Experiments XXIV and XXVII) may fail to digest in the presence of alkali, failure to digest indicating absence of leucoprotease. In association with this fact, it is noteworthy that after aspiration of fluid, resolution of fibrin is incomplete, and at the end of ten days or two weeks, fibrin is much more abundant when fluid has been withdrawn than when pleurisy has pursued an interrupted course.

It is by no means improbable that the localized abscesses which have been found more frequent after aspiration than with uninterrupted pleurisy are referable to a similar cause, namely, increased autolysis no longer restrained by anti-enzyme of the exuded fluid. Enzyme set free by autolysis of cells, on the one hand, may be rapidly removed or destroyed, whereas, on the other hand, when such products are present in large quantity or when their escape is prevented, a localized focus of suppuration, characterized by solution of fibrin, may result. In this way, probably, are formed the localized abscesses which occur when fluid is withdrawn after a single injection of turpentine. Nevertheless, though focal abscesses have been more frequent after aspiration, they occur when the experimental pleurisy has an uninterrupted course.

Increase of enzyme may be equally efficient in transforming a fibrinous into a purulent exudate. For even though a large quantity of fluid accumulates in the chest after repeated injection of the irritant, continued migration of leucocytes, many of which undergo destruction, will finally set free enzyme (leucoprotease) in quantity sufficient to overcome the large amount of anti-enzyme;

that there is a certain quantitative relation between enzyme and anti-enzyme has been shown in a previous publication.[8] The exuded fluid assumes the character of pus; leucocytes are present in immense number and fibrin infiltrated with leucocytes undergoes disintegration.

The effect of a single injection of turpentine varies within such wide limits that in some instances, doubtless dependent upon peculiarities of the animal injected, one half a cubic centimeter may produce more intense inflammation than two cubic centimeters. The effect of repeated injections is equally variable. In some instances, a sero-fibrinous pleurisy results (Experiments XVII, XXIX, XXX and XXXI). It is noteworthy that removal of fluid is followed by transformation of such a sero-fibrinous into a purulent exudate, even though a large part, if not all of the turpentine which still remains in the chest is removed with the aspirated fluid. The serum of the purulent exudate fails to inhibit in vitro the enzyme contained in the fibrin, now infiltrated with an immense number of polynuclear leucocytes. Nevertheless in interpreting the experiments which have been described it must be recognized that a sero-fibrinous inflammation cannot in the transitional stages be sharply separated from suppuration.

There can be little doubt that the purulent exudate in which enzyme is more powerful than anti-enzyme itself acts as an inflammatory irritant and intensifies the exudation of fluid and emigration of leucocytes. When after aspiration of fluid and removal with the fluid of all or almost all of the inflammatory irritant, suppuration fails to occur, or is limited to a small abscess cavity (Experiments XXII to XXVI), there is little or no reaccumulation of fluid. With the occurrence of suppuration, on the contrary, there is rapid accumulation of fluid, which gradually increases in quantity and become richer in cells. Immediately after aspiration, if the explanation which has been offered is correct, the disproportion between enzyme and anti-enzyme is greatest, and there is greatest opportunity for solution of fibrin. The unrestrained enzyme, at the same time has an opportunity to exert an injurious influence upon the pleural surfaces.

[8] *Jour. of Exper. Med.*, 1906, viii, 536.

THE DIFFERENTIATION OF STREPTOCOCCI BY MEANS OF FERMENTATIVE TESTS.[1]

By LEO BUERGER, A.M., M.D.

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The question of the identity of streptococci from various sources has given the bacteriologist material for discussion and investigation for many years. Fehleisen[2] and Rosenbach[3] wished to separate the streptococci found in erysipelas from those present in the ordinary local infections, basing their classification upon differences exhibited in the ordinary fluid and solid media. Von Lingelsheim[4] made the morphological distinctions paramount and thus proposed the groups S. longus and S. brevis. More recently Schottmueller[5] suggested a new classification into S. erysipelatos, S. viridans or mitis, and S. mucosus, varieties which were to be recognized by certain distinctive appearances on blood media. Whilst the last method was gaining ground amongst the German workers, English bacteriologists were investigating the fermentative activities of streptococci with a view to ascertaining whether essential differences such as would be of value in classifying could be discovered. Working upon the assumption that the fermentative powers are biological characters of fundamental importance, Gordon,[6] and Andrewes and Horder[7] employed certain fermentable substances in culture media, and grouped the streptococci partly according to their action upon these media and partly according to their sources.

Gordon's nine tests, selected for routine employment were: (1) The clotting of milk in 3 days at 37° C.; (2) the reduction of neutral red broth during an-

[1] Received for publication May 4, 1907.

[2] Fehleisen, Ueber Erysipel, *Deut. Zeit. f. Chir.*, 1882, xvi, 391. Die Ætiologie des Erysipels, Berlin, 1883.

[3] Rosenbach, Mikroörganismen bei d. Wundinfections Krankheiten des Menschen, Wiesbaden, 1891.

[4] v. Lingelsheim, Therapie und Ætiologie der Streptokokken-Infectionen Berlin, 1899.

[5] Schottmueller, *Münch. med. Woch.*, 1903, l, 849.

[6] Gordon, *Lancet*, 1905, lxxxiii, 1400.

[7] Andrewes and Horder, *Lancet*, 1906, lxxxiv, 708.

aërobic incubation for 2 days at 37° C.; the production of an acid reaction in aërobic cultures 3 days old at 37° C. when cultivated in various slightly alkaline broths containing severally 1 per cent. of the following: (3) saccharose; (4) lactose; (5) raffinose; (6) inulin; (7) salicin; (8) coniferin; (9) mannite.

Andrewes and Horder, making use of these tests and reviewing the results of other workers propose the following very elaborate classification of streptococci.

A. *Streptococcus equinus:* a saprophytic group in which most of the organisms ferment only saccharose and the glucosides.

B. *Streptococcus mitis:* saprophytic, in the human saliva and fæces fermenting saccharose and lactose and not the glucosides.

C. *Streptococcus pyogenes:* long chained, hemolytic, acidify milk, do not clot milk; ferment saccharose, lactose and salicin. Any of the three "cardinal" reactions may be suppressed. Certain other reactions may be added.

D. *Streptococcus salivarius:* characteristic; fermentation saccharose, lactose and raffinose.

E. *Streptococcus anginosus:* cannot always be separated from Class D; occurs in the throat; in chemical reactions practically indistinguishable from *S. salivarius.* Type-reactions are: clotting of milk, reduction of neutral red, acid formation in saccharose and lactose and often in raffinose.

F. *Streptococcus fæcalis.*

G. *Pneumococcus.*

Working along similar lines in 1904, I arrived at conclusions so different from those embodied in the recent paper of Andrewes and Horder that I think it desirable to present them in this paper.

TECHNIC.

Fermentation experiments were made with thirty-three strains of pathogenic streptococci obtained from the human body, and upon one non-pathogenic variety isolated from milk. The source of the organisms is given in the table.

1. Empyema.
2. Mastoiditis.
3. Streptococcæmia-blood.*
4. Cellulitis of foot.
5. Empyema.
6. Mouth.†
7. Liver abscess.
8. Cerebro-spinal fluid-meningitis.
9. Peritonitis.
10. Pelvic abscess.
11. Retroperitoneal abscess.
12. Suppurative arthritis; elbow.
13. Pyonephrosis.
14. Appendicitis.
15. Osteomyelitis; femur.
16. Osteomyelitis; metatarsal.
17. Cellulitis.
18. Streptococcæmia-blood.*
19. Diarrhœa; fæces.
20. Diarrhœa; fæces.
21. Diarrhœa; fæces.
22. Diarrhœa; fæces.
23. Diarrhœa; fæces.
24. Streptococcus enteritis; fæces.‡
25. Metritis.
26. Diarrhœa; fæces.
27. Milk.
28. Peritonitis.
29. Mouth.
30. Streptococcæmia; blood.*
31. Cellulitis.
32. Mouth.
33. Mouth.
34. Mouth.

* Isolated by blood culture in cases of "malignant endocarditis."

† The mouth streptococci were virulent for white mice.

‡ Through the kindness of Dr. Libman.

It was deemed important to determine the nature of the medium most favorable for the growth of the organisms. A sugar-free broth served as a basis and litmus (Kahlbaum's) was added to serve as an indicator. The results obtained showed in a number of instances that carbohydrates apparently unaffected in one medium could be easily split up in another. Therefore trials were made with the fluid media at various titers, with and without the addition of ascitic serum, and also in a medium containing beef serum and water. The following is the list of the media employed.

1. Sugar free broth	0.8 per cent. acid.
2. " "	0.5 per cent. acid.
3. " "	neutral.
4. " "	0.5 per cent. alkaline.
5. " "	0.5 per cent. acid plus one third volume ascitic fluid.
6. Beef serum and water.	

To these media one per cent. of the following carbohydrates was added.

Pentoses: arabinose, rhamnose.

Hexoses: glucose, levulose, galactose.

Hexahydric alcohols: mannite, dulcite.

Disaccharides: saccharose, lactose, maltose.

Polysaccharides: dextrin, inulin.

Sterilization was conducted on three successive days and was followed by a three-day test in the incubator. The possibility of hydrolytic change in some of the sugars, in the presence of the dilute acid medium, or transformation of some other nature during the process of sterilization had to be considered. Therefore several series of experiments (each repeated twice) were made. Flasks of sugar-free broth with litmus were sterilized, and to these, watery solutions of the various carbohydrates, separately sterilized in large tubes, were added. The amounts in each were so computed that a one per cent. solution of the fermentable substance would result. The contents of the flasks were then filled into tubes and the latter tested in the usual way. It was found that the results obtained in the media thus prepared did not differ from those made up in the usual way.

Three series of experiments were conducted with media 1, 2, 3, and 4; six series with 5 and 6. Daily observations of the cultures

indicated the advantages of the various media and the rapidity of acid formation. At the end of the fourth day the whole set of tubes together with the controls was regularly thrown out after the final readings had been recorded. To wait over a longer period of time for acid production to manifest itself, although preferable on theoretical grounds, seems disadvantageous in practice. If the chemical test is to be of any practical value, its results ought to be final within the specified time.

RESULTS OF THE EXPERIMENTS.

In presenting the results, those obtained upon the most favorable media alone will be given in detail, and the streptococci grouped accordingly. The addition of ascitic fluid not only enhances growth but seems to favor the fermentation of certain of the carbohydrates that were not attacked in the simple media.[8] Careful chemical tests of the serous fluid as to possible glucose or other sugar content were always made in advance. Fermentation tubes containing sugar free broth, litmus and the serum were inoculated with B. coli and examined for acid production. Controls of broth, serum and litmus without carbohydrates were included in the set of tubes inoculated with each strain. Keeping in mind the variations in the quality of ascitic fluid of different patients, it was decided to use a serum from the same source for a complete series of tests on all the organisms.

Inasmuch as arabinose was fermented by but one strain, rhamnose by but three strains, and dulcit (or dulcitol) by none of our series, it may be well to disregard these carbohydrates in an attempt at grouping the organisms. We may then tabulate them according to fermentation of dextrose, levulose, galactose, maltose, saccharose, lactose, inulin, dextrin and mannite, as follows:

	Fermenting all but		Total Number.
1.		S. 18	1
2.	Mannite	S. 3	1
3.	Inulin	S. 1, 4, 6, 7, 11, 19, 20, 23, 31	9
4.	Inulin, Mannite	S. 2, 5, 8, 9, 10, 12, 13, 14, 15, 16, 17, 25, 27, 28, 29, 30, 32, 33, 34	19
5.	Inulin, Lactose	S. 21, 22	2
6.	Inulin, Mannite, Saccharose.	S. 24, 26	2

[8] Libman, *Jour. of Med. Research,* 1901, vi, 84.

In accordance with the above we could distinguish six varieties, those failing to ferment inulin and mannite being in the majority, those fermenting all but inulin being second in number. There were therefore:

22	that did not break up mannite,
32	" " " " inulin,
2	" " " " lactose,
2	" " " " saccharose.

The carbohydrates which are most readily fermented are the hexoses, glucose, levulose and galactose (by all), the disaccharide maltose (by all), and the polysaccharide, dextrin. Acid production usually appears first in maltose, then in levulose, glucose and dextrin. Saccharose and lactose resist the action of a very few streptococci. The following table gives an insight as to the number of streptococci fermenting the various substances.

Number of Streptococci Producing Acid in

Pentoses:	arabinose	1
	rhamnose	3
Hexoses:	glucose	34
	levulose	34
	galactose	34
Disaccharides:	saccharose	32
	lactose	32
	maltose	34
Hexahydric alcohols:	mannite	12
	dulcite	0
Polysaccharides:	dextrin	34
	inulin	2

Rhamnose was broken up by S. 19 and S. 23; rhamnose and arabinose by S. 18.

It is important to note that the fermentative properties described suffered no change from generation to generation. All the organisms were studied over a period of from six to eight months, an interval of at least six months having elapsed between the first and the final tests. The results on the serum media were uniform. This showed that artificial cultivation had no influence upon the fermentative activities.

The Results on Other Media.—Plain broth at 0.8 per cent. acid, 0.5 per cent. acid, neutral, or 0.5 per cent. alkaline titer were found to be inferior to the serum bouillon. At the 0.8 per cent. titer a number of organisms failed to attack carbohydrates which were

easily broken up in the presence of serum. These were mannite (not affected by 3 strains), lactose (2 strains), dextrin (2 strains), and saccharose (1 strain). The 0.5 per cent. acid plain broth was more favorable and fell behind the serum medium only in the case of lactose (1 strain), saccharose (1) and mannite (3). Neutral bouillon gave the same results as the last, but growths were more luxuriant. The alkaline 0.5 per cent. medium was rejected as unsuitable both because moderate acid production was indicated with difficulty, and because this titer was unfavorable for growth. Beef serum-water as suggested by Hanna[9] and Hiss,[10] although a good culture medium, was found unreliable. The results in it were variable, and frequently a streptococcus would fail to make acid from carbohydrates that were easily broken up in the presence of ascitic fluid.

Experiments on the Pneumococcus and Streptococcus Mucosus.—Fifteen strains of the former and eight of the latter were tested. The serum media are even more important for the pneumococcus than for streptococci. In plain broth with carbohydrates growths are frequently so sparse that acid production, if present at all, is too slight to bring about a visible change in the indicator. All the pneumococci and streptococci of the "mucosus" variety gave the same fermentative reactions thus differing from the ordinary streptococci. Acid was formed from dextrose, dextrin, maltose, lactose, galactose, inulin, levulose and saccharose. They failed to break up mannite, dulcit, arabinose and rhamnose.

A much larger series of pneumococci (65 strains) were tested for their power to ferment inulin. It was found that a number of them failed to produce acid in certain generations. All the organisms, however, fermented inulin at some time or other of their life history.

More recently in a study of pneumococci obtained from blood cultures it has been found by Dr. Ryttenberg and myself that the power of breaking up inulin may be lost. These observations on the mutability of fermentative properties will be published later.

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[9] Hanna, *Jour. of Path. and Bact.*, 1898, v, 267.
[10] Hiss, *Jour. of Exper. Med.*, 1904. vi, 317.

SUMMARY.

If we glance again at the classification proposed by Andrewes and Horder we are struck at once by the fact that no hard and fast differences in fermentative properties characterize the various groups, and that the authors prefer to have recourse to the most frequent habitat of the organisms and to chemical tests, rather than to confine themselves to the latter method alone. From what has been said earlier in this paper, it becomes clear at once that the results gained by these authors are not convincing, for in their work they have failed to use the most favorable medium for growth of the organisms. And thus it can hardly be doubted that different reactions might have been obtained, under the conditions adopted by us. How otherwise could the fact be explained that of thirty-four of their pneumococci only eight fermented inulin. We had a similar experience while using the plain broth which led us to reject it as an unfavorable medium. Further, all our pneumococci fermented lactose with great rapidity. Andrewes and Horder report that eight of their series left lactose unchanged.

Taking our own tests into consideration we find that working with but a small number of carbohydrates we were able to find six different varieties of streptococci among only 33 pathogenic strains. Which of the various substances is to decide us in the grouping of the organisms? Would we not find even greater variations from the most common type (see No. 4 in table) if we were to extend our tests over a larger series of chemical agents? These questions are difficult to answer and only extended experimentation with a great many streptococci and many media will clear up the doubtful points.

In concluding it may be stated:

1. Streptococci vary considerably in their ability to produce acid from various carbohydrates.

2. Chemical tests of this kind should be made only in the media which are most favorable for the growth of the organisms.

3. Our results gave us six groups of streptococci, when tested upon dextrose, levulose, galactose, maltose, saccharose, lactose, inulin, dextrin and mannite, viz.: Those fermenting (1) all; (2) all but mannite; (3) all but inulin; (4) all but inulin and mannite;

(5) all but inulin and lactose; and (6) all but inulin, mannite and saccharose.

4. In view of the comparatively small number of streptococci employed we are hardly warranted in making a definite classification. Perhaps a larger series of tests upon the media employed will enable us to divide streptococci into distinct classes characterized by certain fixed fermentative properties.

ON EXTRACELLULAR AND INTRACELLULAR VENOM ACTIVATORS OF THE BLOOD, WITH ESPECIAL REFERENCE TO LECITHIN AND FATTY ACIDS AND THEIR COMPOUNDS.[1]

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INTRODUCTION.

Snake venom forms a hæmolytic compound with pure lecithin when it is shaken with a chloroform solution of this substance.[2] The same is true of the venom of bee.[3] Judging from this important discovery, linked with the fact that the serum as well as the corpuscles of blood yield upon hot alcoholic extraction a large quantity of lecithin, Kyes was led to deduce that the venom activating substance of blood is lecithin. This theory has, however, some rather serious difficulties in explaining certain fundamental phenomena which are observed in venom hæmolysis. In spite of the fact that all serums or corpuscles do yield upon extraction nearly uniform amounts of lecithin, certain kinds of serums possess no activating property; and the susceptibility of the corpuscles of different bloods exhibits a wide variation according to the species of animals. In some instances the corpuscles are completely refractory to the action of venom. Speaking quantitatively there is no relation between the amount of lecithin and that of the venom activating substance of serum or corpuscles. If we are forced to hold lecithin responsible for venom hæmolysis in normal serum or blood corpuscles we must assume that lecithin exists in different states in different bloods, the explanation adopted by Kyes to account for the phenomena observed.

This hypothesis appears to furnish an easy solution of the phenomena, but it requires, in the meanwhile, its verification by experi-

[1] Received for publication April 15, 1907.

[2] Kyes, *Berl. klin. Woch.*, 1903, xi, 956, 982.

[3] Morgenroth and Carpi, *Berl. klin. Woch.*, 1906, xliii, 1424.

mental evidence. The first question will be whether lecithin ever exists as an available compound in the activating serum and in the venom-susceptible corpuscles. To decide this point it is absolutely necessary to exclude other activators than lecithin from the activating serum or susceptible corpuscles. This having been done, we can compare the degree of firmness of lecithin-containing compounds of various kinds of serums and corpuscles, including the originally activating and non-activating serums and corpuscles. Even if we find that in one set of serums there is present an available lecithin compound, and not in the other, we shall then face a new problem, namely, whether the fact of availability or the looseness of the lecithin compound in the former serums is applicable to the whole blood or is to be attributed to a particular lecithin compound found only in the serum, but not in the blood corpuscles.

The part which lecithin may play in venom hæmolysis caused by fresh serum is not quite clear, inasmuch as serum contains still other complementary substances, which, doubtless, play their own rôles. A sharply differential method for venom hæmolysis caused by lecithin and by certain other complementary substances is required to clear up the question to what extent lecithin is responsible for the production of hæmolysis by venom in unmodified serums.

Before recording the results of my present investigations relating to the points already referred to above, as well as to some other questions to be introduced and considered later in this paper, a brief statement of the recent development of our knowledge concerning venom hæmolysis will make the necessity and object of the present work more obvious.

Snake venom produces hæmolysis only through the aid of a secondary substance. Thus Flexner and Noguchi[4] first demonstrated that the blood corpuscles of certain species of animals undergo venom hæmolysis when there is present suitable serum, and believed that complement of such serum is the activating principle. This was, however, found not to be always the case, as Kyes[5] discovered that there are instances where the corpuscles are dissolved without the addition of serum. The cause of the independent susceptibility of these corpuscles was ascribed to the presence of intracellular activator in these cells. The general characteristics of intracellular activators agreed with those of serum complement; Kyes called them endocomplements. At the same time lecithin

[4] Flexner and Noguchi, *Jour. of Exper. Med.*, 1902, vi, 277.
[5] Kyes, *Berl. klin. Woch.*, 1902, xxxix, 886, 918.

was found to be capable of activating venom with striking readiness, but Kyes distinguished this form of venom hæmolysis from that caused by the complementary substances of certain fresh serums or by the endocomplement of certain susceptible corpuscles, by their resistance to high temperature and their activity at 0° C., in which lecithin excelled the other. He also confirmed the important observation of Calmette[6] of the acquisition of power or the increase in strength of the venom activating property of various serums after heating to 65° C. and higher. This phenomenon was attributed by Keyes to the liberation of lecithin by the action of heat. While adding still further evidences to the occurrence of venom-complement hæmolysis, Keyes and Sachs[7] withdrew the opinion that endocomplement was a thermolabile complement, and presented a new view that it is really lecithin contained in the stroma of the susceptible corpuscles. The reason why the stroma of venom-resistant corpuscles does not react with venom was explained by the hypothesis that lecithin exists in an unavailable form in these corpuscles. In his third communication Kyes[8] succeeded in preparing a compound of venom and lecithin (lecithid) and threw suspicion on that mode of venom hæmolysis in which complement is thought to take a part.

Kyes suggested the possibility that any injurious substance may modify the corpuscles so as to render available the lecithin otherwise inaccessible to the venom. But this assumption has never been proven experimentally. My previous experiments[9] show lecithin to be by no means an inert compound, although its activity is likely to be underestimated on account of the slow reaction-time. Further, I was led to consider certain oleic compounds as well as oleic acid as venom activating agents.[10] This point has an important bearing on the present investigation of the venom activating substances of normal serum and susceptible corpuscles. Although no relationship between the lipolytic and the hæmolytic properties of venom and phytogenous toxalbumens have been established, yet the discovery by Neuberg and Rosenberg[11] of the lipolytic property of various venoms and bee poison is a fact of great interest. Ricin, which is a powerful lipolyzer,[12] forms a strong hæmolysin when mixed with free lecithin,[13] a fact important in that it shows that ricin does not unite with the lecithin in the integral corpuscles or serum. Apparently lecithin in the native condition in these substances is unattackable by ricin.

Hence it appears that we are again in the dark as to the real nature of venom hæmolysis. We are entirely unable to answer the question whether complement and certain complement-like bodies of serum have any part in venom hæmolysis, and whether lecithin

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[6] Calmette, *Compt. rend. d. l'Acad. d. Sciences*, 1902, cxxxiv, 1446.

[7] Kyes and Sachs, *Berl. klin. Woch.*, 1903, xl, 21, 57, 82.

[8] Kyes, *Berl. klin. Woch.*, 1903, xl, 956, 982.

[9] Madsen and Noguchi, Oversight over det Kongl. Danske Vedenskabernes Selskabs Forhandlungen, 1904.

[10] Noguchi, *Jour. of Exper. Med.*, 1906, viii, 87.

[11] Neuberg and Rosenberg, *Berl. klin. Woch.*, 1907, xliv, 54.

[12] Pascucci, *Hofmeister's Beiträge*, 1906, vii, 457.

[13] Neuberg and Rosenberg, *loc. cit.*

is really present in available form for venom in activating serum and susceptible corpuscles. The sum of what we know at present is that blood serum and corpuscles yield large amounts of lecithin upon alcoholic extraction, that venom can form a powerful hæmolytic compound with free lecithin, that the activating property of serum and venom susceptibility of the corpuscles have no direct relation to their lecithin content, that certain activating serums contain venom activating principles which in some respects closely resemble complement, that certain chemicals, which may be present only in certain bloods as normal constituents, can produce a form of venom hæmolysis hardly to be distinguished from that caused by normal serum.

A ready way to clear up this confusing point is to discover an agent possessing the elective inhibitory action upon one or other of the venom activating principles. In course of my study[14] on the *anti*complementary action of various acids, alkalies and salts, I found, among others, that various salts of the alkali earths inhibit complementary action without altering the serum amboceptors. Calcium chloride,[15] when used in a dilute solution, is most suitable to remove the complementary action of serum. This salt was employed in the present work to inactivate serum complement, as it has no marked destructive action upon venom amboceptor, when used in a strength of $\frac{1}{10}$ N, or weaker. On the other hand, calcium chloride has no anti-activating power against lecithin. Venom lecithid retains its hæmolytic activity in a medium containing calcium chloride. When the amount of lecithin, or venom lecithid, is very small the salts retard complete hæmolysis, but have no ultimate effect on the process.

In addition to this differential agent an ethereal extraction of venom activators prepared from an active serum or the stroma of susceptible corpuscles was employed as a means to distinguish the lecithin-like activator from the complement-like ones. This method was especially useful for determining the protein-lecithid nature of the activator of certain serums and all heated serums. I shall return later to a discussion of this point.

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[14] Noguchi, read at the meeting of the American Society of Bacteriologists, New York, Dec. 28, 1906.

[15] 0.85 per cent. of this salt is isotonic.

VENOM ACTIVATORS OF FRESH SERUM.

To determine whether the venom activating property of fresh serum is removed by calcium chloride, 0.5 c.c. of the serum under consideration was mixed with 0.5 c.c. of $\frac{1}{10}$ N. solution of this salt, and the volume made 2 c.c. with 0.9 per cent. salt solution. The washed corpuscles were added in proportion of five per cent. After an incubation of half an hour at 37° C., 0.1 c.c. of 0.4 per cent. solution of cobra venom was introduced, and the whole incubated for several hours at 37° C. and left at room temperature for the rest of the night. The result was read off, as a rule, within twenty-four hours. The corpuscles and serum came from the same blood; but sometimes the washed corpuscles of a second blood were employed. This last combination is, of course, possible only when the serum in question has no hæmolytic action upon the corpuscles. Table I. shows the results obtained with the serum and corpuscles of the same blood.

TABLE I.

Blood of	Control. Fresh Serum 0.5 c.c. + Corpuscles. + 0.9 % NaCl 1.5 c.c. } 0.1 c.c. of 0.1 % Cobra Venom Solution.	$CaCl_2$ Addition. Fresh Serum 0.5 c.c. + Corpuscles. + N/10 $CaCl_2$ 0.5 c.c. + 0.9 % NaCl 1.0 c.c. } 0.1 c.c. of 0.1 % Cobra Venom Solution.
Man	complete hæmolysis	no hæmolysis
Horse	"	"
Pig	"	"
Cat	"	"
Guinea-pig	"	"
Rabbit	"	"
Pigeon	"	"
Hen	"	"
Goose	"	"
Goat	no hæmolysis	"
Ox	"	"

The above experiment shows that the majority of the varieties of blood employed undergo complete hæmolysis by venom and that their activating substances are completely inhibited by calcium chloride. The absence of hæmolysis in the presence of $CaCl_2$ is not due to destruction of the venom, because the addition of a small quantity of free lecithin to such mixture produces complete hæmolysis. Or, the presence of a small amount of lecithin, which may be added purposely at the same time as the $CaCl_2$, leads to complete hæmolysis. There is still another way to prove that the corpuscles are sensitized by venom in the $CaCl_2$ mixture. The corpuscles may

be washed with 0.9 per cent. salt solution and finally suspended in a fresh lot of the latter. If now a small amount of lecithin or 0.5 c.c. of any activating serum is added complete hæmolysis occurs.

Whether or not the venom activators of the serums are identical with compounds contained in them cannot be determined from this experiment, as both are inactivated by $CaCl_2$. At all events, lecithin is excluded by this test as the activating agent of these serums.

The serum of the dog is extremely rich in venom activating substances and differs from other serums in its relation to inactivation with calcium chloride. Of twelve different samples of normal dog serum complete inactivation by $CaCl_2$ was obtained only in three, marked delay, in five, and slight retardation, in the rest. In this series homologous and heterogeneous corpuscles were tested and gave the same result. Thus dog's serum is an example of a fresh serum, in which lecithin exists in an available form for venom activation. The simultaneous presence of certain $CaCl_2$ inhibiting venom activators was also demonstrable; and in a few instances this class of activators alone was present in the dog serum.

VENOM ACTIVATORS OF BLOOD CORPUSCLES.

A wider variation is exhibited by the corpuscles of different species of animals in their action toward venom. Some kinds of corpuscles are promptly, while other not at all dissolved by venom. The latter class of corpuscles remains undissolved because of the absence of suitable venom activators although sensitization still occurs. The corpuscles of all bloods undergo hæmolysis when suitable activators are present. The most susceptible corpuscles are those of dog and guinea-pig, and entirely refractory are those of ox, goat and sheep. The corpuscles of horse, rabbit, rat, pig and man occupy an intermediary position. Those which approach the limit of the series of non-susceptibility require a longer incubation for hæmolysis than those which stand near the opposite end. This incubation time can be greatly shortened by adding certain activating serums. The original assertion of Flexner and Noguchi[16] that venom requires complement for its activation was based on the fact that certain washed corpuscles remained intact

[16] Flexner and Noguchi, *Jour. of Exper. Med.*, 1902, vii, 277.

during a period of experimentation in which the same corpuscles underwent complete hæmolysis when fresh serum was added. In a later paper Flexner and Noguchi[17] described the presence of complementary substances in various kinds of organic cells.

The cause of destruction of certain corpuscles by venom alone is ascribed by Kyes[18] and Kyes and Sachs[19] to the presence of endocomplement. The thermolabile nature and the inactivity at 0° C. of endocomplement led them first to classify it with complements. But later they showed that the thermolability is caused by the simultaneous presence of hæmoglobin and hence concluded that it was the lecithin of the stroma. To this particular point I will return a little later.

In my experiment regarding the nature of intracellular venom activators of the integral corpuscles, I employed calcium chloride to determine whether this salt can suppress hæmolytic action of venom upon susceptible corpuscles. The result was rather remarkable. The washed corpuscles of horse, rat, rabbit, cat, guinea-pig, pig, pigeon, goose, hen and man remained undissolved in $\frac{1}{10}$ N. to $\frac{1}{100}$ N. $CaCl_2$ medium in the presence of corbra venom. The controls dissolved completely in from fifteen minutes to twenty-four hours. The protection of the susceptible corpuscles is not due to the desctruction of venom by $CaCl_2$, because by a later addition as well as by the simultaneous introduction of a small amount of lecithin, or even by the addition of old dog serum (inactive with age) complete hæmolysis can be induced. The least susceptible corpuscles require the smallest amount of $CaCl_2$ for protection. On the other hand, a trace of hæmolysis is often observed with the corpuscles of guinea-pig or man after twenty-four hours. Different samples of dog corpuscles behave differently. I employed several samples and with some there is perfect protection, with others partial protection. Dog corpuscles are liable to spontaneous hæmolysis within twenty-four hours, which fact may be responsible for irregular results.

The removal of intracellular activators by means of digestion

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[17] Flexner and Noguchi, *Jour. of Path. and Bact.*, 1905, x, 111.

[18] Kyes, *loc. cit.*

[19] Kyes and Sachs, *loc. cit.*

in a calcium chloride solution gave variable results. The corpuscles of horse or pig are protected from venom after digestion in $\frac{1}{60}$ N. $CaCl_2$ for half an hour, but those of guinea-pig and dog are not. If we use $\frac{1}{10}$ N. solution these corpuscles are rendered even more susceptible to venom than the undigested samples. The mechanical injuries brought about during the procedure of washing away the calcium salt with sodium chloride solution may have some influence. Perhaps a sudden change of tonicity and breaking up of agglutinated masses of corpuscles hasten the destruction. Hence it would appear that in the majority of the corpuscles lecithin seems not to be able to play a part as activator. Before deciding this matter finally other considerations should be taken up. The presence of hæmoglobin in lecithin containing fluid, or the addition of lecithin to the corpuscular solution does not permit of the inactivation by calcium chloride. Ovovitellin of hen's egg contains a high percentage of lecithin. This lecithin-proteid is not split up with ether, but by hot alcohol. I prepared a saline suspension of ovovitellin and examined it for venom activation. It was found to be an excellent activator. Hence the question arose whether this proteid compound could not be easily inactivated by calcium chloride. This proved not to be possible. Thus lecithin, either in a mechanical mixture with hæmoglobin or corpuscular contents, or in a chemical combination with proteid, is not inactivated by $CaCl_2$. That lecithin does not exist in an available form for venom hæmolysis in susceptible as well as insusceptible corpuscles can be shown by the following experiments.

The washed corpuscles of the guinea-pig were broken up with about three times their volume of water. The stroma was separated out by adding 0.9 per cent. sodium chloride and the precipitated stroma was collected by centrifugation. The clear, intensely dark-red supernatant fluid was pipetted off and used for some tests, while the stroma was twice washed in a 0.9 per cent. sodium chloride solution.

The pink-colored stroma was tested for its venom activating property, for which the corpuscles of ox and goat were used. The activation was not rapid, but progressed rather slowly. The deep-red supernatant fluid was not activating. The test with calcium chloride showed that the activating property of the stroma is completely removed by this salt. The shaking of the stroma with a large quantity of ether also removed this property. On the other hand, the ethereal extract upon evaporation left a small amount of fatty substances, which contained chiefly aceton-soluble fats, but almost no lecithin to be detected even with venom.

I redissolved the oily mass in 0.9 per cent. salt solution, in which it showed turbidity and an acid reaction to litmus. This suspension is activating.

We have thus succeeded in locating the site and identifying the probable nature of the phosphorus-free fat acting as an intracellular venom activator. It may be added that the activating property of this suspension is completely set aside by calcium chloride. As Kyes and Sachs first showed, lecithin could be isolated from the stroma with hot alcohol. The lecithin obtained from it is activating, but entirely different from the activators of the integral corpuscles, corpuscular solution and ethereal extract of corpuscles in its relation to $CaCl_2$. While lecithin can be extracted with alcohol from any of the corpuscles, no matter whether they belong to the susceptible or the insusceptible class, in nearly equal quantities, the ether-soluble venom activators are found only in the corpuscles susceptible to venom. I have tried many times to extract an ether-soluble venom activator from the corpuscles of the ox and goat, but without success. These corpuscles are naturally venom-resistant, and their corpuscular solutions do not contain a venom activator. A later experiment with the corpuscles of the dog gave practically the same result as those of guinea-pig.

Direct evidence that certain fatty acids, soaps and neutral fats are capable of acting as venom activators can easily be given. The venom-resistant corpuscles are washed and freed from the serum. Then the minimal hæmolytic quantities of oleinic acid, sodium oleate, ammonium oleate, neurin oleate and triolein are ascertained. The subminimal hæmolytic dose of any of these chemicals is taken and mixed with the corpuscular suspension of any of the insusceptible bloods. No hæmolysis occurs after twenty-four hours. But if an adequate quantity of cobra venom is added at the same time, complete hæmolysis occurs. The addition of these chemicals to the corpuscular solution (not suspension) of an insusceptible blood renders it venom-activating. This artificially prepared activating solution of blood corpuscles behaves in the manner of the solution of susceptible corpuscles, and becomes inactive upon ethereal extraction in case of oleinic acid, organic soaps, and triolein. With the solutions whose activating property is conferred by the addition of alkaline oleate soaps, ether fails to remove it. Calcium

chloride is very effective in depriving these solutions of their acquired venom-activating property. The result with triolein is not satisfactory, as two preparations from Merck and Kahlbaum gave entirely different results, as the latter was almost entirely inactive. The activators of susceptible corpuscles were completely and permanently inactivated by boiling.

From these results it seems justifiable to conclude that lecithin does not exist in the corpuscles, irrespective of their susceptibility to venom hæmolysis, in an available form for venom. The degree of susceptibility of blood corpuscles depends chiefly upon the amount of ether-soluble activators contained in the cells. These ether-soluble activators are, doubtless, fatty acids, and especially oleinic acid. The absence of fatty acids in the insusceptible corpuscles is in perfect harmony with the reaction to venom. If we compare the amount of fatty acids[20] and the degree of venom susceptibility of different corpuscles we discover that a definite and undeniable parallelism between these two factors exists. If any amount of venom activators are present in the stroma after ethereal extraction it does not necessarily follow that they are lecithin, because the ether-insoluble soaps may be preëxistent or be formed during the manipulation of the corpuscles with water, and they would certainly take on a good share in the activation.

VENOM ACTIVATORS OF HEATED SERUM.

Calmette discovered that any serum acquires venom activating property or has its property increased by heat above 65° C. Kyes found that the maximum effect is reached at 100° C. and coincides with the maximum liberation of lecithin. That the activating property of such heated sera is due to lecithin can be further established by means of chloride of calcium, as the next table shows.

An attempt was made to ascertain whether lecithin exists in a free state or as a proteid compound in heated serum. That a non-coagulable proteid exists in serum heated to 80° to 100° C. in a neutral or slightly acidified reaction has long been known. Chabrié,[21] who first described this proteid, called it albumon. Howell's[22]

[20] Abderhalden, *Zeit. f. Physiol. Chem.*, 1898, xxv, 65.
[21] Chabrié. *Compt. rend. de l'Acad. de Sciences,* 1891, cxiii, 557.
[22] Howell, *Amer. Jour. of Physiol.*, 1906–7, xvii, 280.

TABLE II.

	Serum 0.2 c.c. Corpuscles 5 per cent. Cobra Venom 0.1 per cent., Solution 0.1 c.c. (added later). } Total Volume made to 2 c.c. with 0.9 per cent NaCl Solution.									
	Fresh.		56° C.		65° C.		85° C.		100° C	
	Saline.	$CaCl_2$ N/10 0.5	Saline.	$CaCl_2$ N/10 0.5	Saline.	$CaCl_2$ N/10 0.5	Saline.	$CaCl_2$ N/10 0.5	Saline.	$CaCl_2$ N/10 0.5
Man	C. H.	None	Slight H.	None	Much H.	Moderate	C. H.	C. H.	C. H.	C. H.
Horse	"	"	"	"	"	Much H.	"	"	"	"
Guinea pig	"	"	C. H.*	"	C. H.	C. H.	"	"	"	"
Ox	None	"	None	"	Slight H.	Slight H.	"	"	"	"
Goat	"	"	"	"	"	"	"	"	"	"

recent investigations of this proteid added many new facts. Albumon is characterized as soluble in water, indiffusible through collodium membrane, non-coagulable in presence of neutral or acid reaction upon boiling, but easily precipitable by half saturation with ammonium sulphate. It contains a large percentage of phosphorus and iron. The phosphorus represents the lecithin which exists in its molecule as a component extractable only by warm alcohol. Howell considers albumon an artificial product formed through the action of the heat, while some previous investigators considered it a normal constituent, or were uncertain on this point.

In my experiments I employed ox serum. The serum was diluted with three times its volume of water and gradually boiled at neutral reaction (neutralization by means of acetic acid). The non-coagulable portion was separated from the coagulum by filtration. The clear fluid gave the required characteristics for albumon, and upon examination it proved to be highly active for venom and not to be inactivated by calcium chloride. Ether failed to extract any activator from the fluid medium or after drying. On the other hand, warm alcohol yielded much lecithin and perhaps some other venom activating substances. The proteid precipitated out by the process of extraction was inactive. Goat serum yielded a similar albumon.

Thus the conclusion seems warranted that the lecithin of heated serum does not exist in a free state, but in a proteid compound, which is capable of reacting with venom. In this case the preexistence of this proteid in unheated serum is conclusively excluded by the fact that neither the fresh serum, nor the coagulum of these bloods contained any available lecithin compounds.

* Guinea-pig corpuscles contain endocomplement, which accounts for complete hæmolysis. If ox or goat corpuscles are used no hæmolysis results, while the same corpuscles are readily dissolved by the addition of over-heated serum, and cannot be reduced by $CaCl_2$.

LOCATION OF AVAILABLE LECITHIN IN VARIOUS PROTEID FRACTIONS OF SERUM.

The existence of venom activators of lecithin nature in normal dog serum has been mentioned. But the question whether the availability of lecithin in this particular serum is due to looseness of a lecithin-proteid compound, or to the presence of a definite lecithin compound peculiar to this serum was left undecided.

Two different samples of dog serum were dialyzed in collodium sacs against running water and after seventy-two hours the whitish precipitates were collected by filtration and dissolved in 0.9 per cent. sodium chloride solution. The filtrate was made isotonic with the same salt. The first represents the serum globulin and the second the serum albumin. These were now tested for venom activating property. The result shows that the globulin fraction was inactive and the albumin highly active. The same serums were then fractionated with ammonium sulphate. The precipitate produced by half saturation was separated from the serum by filtration. The filtrate was then completely saturated by further addition of this salt. The precipitate was obtained by filtration. The first represents the globulin and the second the albumin fraction of the serum. Both were dialyzed in collodium sacs for seventy-two hours. In the first sac, which contained the globulin fraction, there was a large amount of whitish precipitate. This was filtered and collected; the filtrate was also preserved for testing. The content of the second sac was perfectly clear, and was used for the tests. All three were made isotonic with sodium chloride. The globulin precipitate dissolved in this medium with slight opalescence. The tests for venom activation gave the following result: the solution of the globulin precipitate and the albumin fraction were unable to activate venom, while the clear portion of the first sac was extremely active. This last fraction was non-coagulable in saline solution upon boiling, but precipitable by ammonium sulphate and heat and coagulable by alcohol. It gave a blue-violet Biuret reaction. The venom activating property of this portion was unaffected by $CaCl_2$ or ethereal extraction.

Similar tests with ox and pig serums (both non-activating) gave results differing from those with dog serum, as the globulin lacked the venom activating property.

The next question was whether the globulin and albumin fractions of these serums contain any lecithin. The examination was made by extracting the serums with hot alcohol (70° C.).

The globulin fraction of the serums of dog, horse, ox and pig yielded upon hot alcoholic extraction comparatively small amounts of lecithin. On the other hand, the albumin fraction of these serums yielded much more lecithin than the globulin. The non-coagulable portion obtained from the first sac (containing the pre-

cipitates by half saturation with ammonium sulphate) contained a large amount of lecithin.

The globulin as well as albumin fractions did not produce upon boiling, either with or without sodium chloride, as much lecithin (in available form for venom) as the whole serums from which they were isolated. In some instances even considerable reductions were observed with the globulin fraction plus the non-coagulable lecithin proteid of dog serum. I am inclined to ascribe this reduction in activity to the simultaneous presence of cholesterin in the medium. The problem, which was set at the beginning of this topic, has been resolved experimentally: the venom activating substance of lecithin nature in dog serum exists as a definite proteid compound, and does not depend upon a loose combination between lecithin and globulin or albumin. The non-venom-activating serums do not contain a similar lecithin proteid to that found in dog serum.

PREPARATION OF ARTIFICIAL VENOM ACTIVATING SERUM.

Normal serum may contain two different sets of venom activators, namely, one resembling complement and the other a proteid compound of lecithin resembling albumon. The latter is occasionally present in normal dog serum, while the former constitute the venom activating substances contained in all venom activating serums. Calcium chloride renders the first class of activators ineffective. The venom activating property of these serums, which are subject to the calcium inactivation, is completely or in part removable by ethereal extraction. The ethereal extract contains fatty acids and neutral fats, but not lecithin. The inactivation of these serums by the temperature of 56° C. is rather uncertain, although more or less reduction in activity is observed. Sometimes complete loss is obtained. The activating function of this class of venom activators is suspended at 0° C. Except in certain herbivorous mammalian bloods (ox, goat and sheep) the majority of mammalian serums and certain avian serums contain the complement-like venom activators in varying quantities, and no lecithin-like activators are to be found in these serums. Even in dog serum, which contains an available compound of lecithin, there is

a large amount of the other present. I have already mentioned that in the ethereal extract of these serums varying quantities of fatty acids and fats are contained. But more convincing is the fact that none of the non-activating serums yields more than a minute quantity of activating fatty acids.

By the term " venom activating fatty acids " oleic acid is chiefly meant. Palmitic and stearic acid are far less effective in this respect. The more oleic acid is present in the ethereal extract, the stronger is the venom activating property.

Apart from the varieties of the fatty acids, the whole amount of these acids extractable from dog serum is nearly twice as much as from the serum of ox, goat or sheep.[23] I have already mentioned that two samples of triolein which I used acted very differently, so that I have been put in doubt regarding my earlier experiments.[24]

The facts above enumerated clearly indicate that fatty acids have a direct relation to the venom activating property of blood serum.

Leaving aside the question whether fatty acids represent the entire venom activators or only part of them, I will consider next whether or not certain fatty acids and soaps can confer the venom activating property upon non-activating serums.

For this experiment two sets of normal ox serum were used: the one without any modification, and the other after being shaken with a large volume of ether. To both sets oleinic acid in amounts of 0.12[25] to 100 grams of the serum was added. In still other series, and with ox serum, sodium oleate was used instead of oleinic acid. Oleinic acid and sodium oleate are highly hæmolytic if these are dissolved in this concentration (or emulsified in case of oleinic acid) in a 0.9 per cent. sodium chloride solution; but in ox serum they remain completely inactive. When these mixtures are used as venom activators they display very powerful hæmolytic action upon insusceptible corpuscles (ox and goat). Their action is not so prompt as that of dog serum or any heated serum, but more like that of guinea-pig serum, except that it is prompter than that. By

[23] Abderhalden, *loc. cit.*

[24] Noguchi, *Jour. of Exper. Med.*, 1906, viii, 87.

[25] This proportion was taken from the data given by Abderlhalden for dog serum.

reducing the quantity of the mixture a descending scale of activation can be secured until no effect whatever is obtained. There is no marked difference between the acid and soap in the mode of action. Calcium chloride completely inactivates these mixtures. The temperature of 56° C. has a marked reducing power upon the soap and serum mixture, but hardly any upon the acid and serum mixture. Ethereal extraction removes the activating property of the latter, but not of the former. At 0° C. the latter is still slightly active, but not the former.[26]

These artificially prepared venom activating serums are not easily distinguishable from certain natural serums.

The second type of venom activating serum, in which lecithin is present in an available form, can be artificially prepared, by adding pure lecithin, or the non-coagulable proteid of heated serum, to non-activating serum. When the mixture is made its activating property cannot be stopped by calcium chloride or the temperature of 0° C. Cholesterin inhibits the effect of this mixture. Ether cannot remove much of the lecithin when once mixed with the serum; this perhaps may be due to the fact that lecithin can enter into combination with some of the serum components.[27]

THE PROTECTIVE ACTION OF CALCIUM CHLORIDE AGAINST VENOM CYTOLYSIS.

The powerful cytolytic property of various kinds of venoms upon the cells of liver, kidney, nerve, testis, and ova of different animals has been demonstrated by Flexner and Noguchi.[28] The mechanism of the cytolysis was found to be essentially the same as that of hæmolysis and that intracellular complements played an important part. Knowing no other means to eliminate the intracellular complement at that time, Flexner and Noguchi employed heat (temperature of 55° C. maintained for thirty minutes). They found that this temperature rendered the cells insusceptible to venom, unless fresh serum was added to the mixture. In this way the similarity of venom cytolysis and serum cytolysis was established.

[26] No suppression of action without serum constituents.
[27] Mayer and Terroine, *Compt. rend. d. l. Soc. d. Biol.*, 1907, lxii, 398.
[28] Flexner and Noguchi, *Jour. of Path. and Bact.*, 1905, x, 111.

Since I found in calcium chloride a powerful anti-complementary hæmolytic substance it was natural to expose the somatic cells to the influence of this chemical to establish the nature of the complementary body present in them. In this series of experiments the cells of liver, kidney, testis and brain of the guinea-pig and rat were employed. It was difficult to obtain many ganglion cells in good condition, while the testicular cells were easily obtained intact. Uniform emulsions (5 per cent.) in salt solution of these cells were measured into small test-tubes to which daboia venom was added in proportion of 1 : 100. The mixture was placed in a water thermostat at 37° C. for five hours and examined microscopically. To test the action of calcium chloride the salt was added to the suspensions of these cells in ratio of $\frac{1}{10}$ N., and half an hour later the venom was introduced.

The results are briefly as follows: the cells of the liver, kidney and testis are well preserved in the saline solution after five hours. The ganglion cells are less distinct in outline, but apparently have not disintegrated (controls). In the venom solution the testicular cells are nearly all dissolved, but the spermatozoa minus heads remain. The clearing up of the cellular elements is distinctly visible *in vitro*. The liver cells become more or less swollen and the outlines indistinct. The granules disappear and the nuclei become more distinct. Agglutination of free cells is marked. The kidney cells and tubules become gradually indistinct and a general disintegration of the former occurs. The number of cells is less than in the controls; agglutination occurs. The ganglion cells have disappeared wholly, and a general solution of granular elements has taken place. These alterations are much more pronounced in the emulsions of guinea-pigs' than of rats' brains. Not only the microscopical, but also the macroscopical appearances of the venomized emulsions is at once recognized by a marked clearing up of the original turbid suspensions. The addition of calcium chloride completely prevents the destructive action of venom upon the cells. The protection afforded by this salt is greater than is obtained by heating the cells to 55° C.

From these facts the complement-like nature of the venom activators contained in the somatic cells is once more established.

SUMMARY.

In normal serums of the majority of mammalian and avian blood there exists certain substances capable of activating venom hæmolysin. They are extractable from serum by means of ether, and are capable of conferring upon the originally non-activating serum a power to activate venom, when mixed with the latter. The ethereal extract consists of fatty acids, neutral fats and possibly also some ether soluble organic soaps. The fatty acids and soaps, especially of the oleinic series, acquire certain characteristics of complements in general, when they are mixed with serum. They are inactive without the venom in the mixture; they are inactivable with calcium chloride; they exhibit a tendency to go off in activity with age; they are inactive or only weakly active at 0° C., and they are extractable by ether. In testing the serum from which the ether soluble substances are removed, it is found that no venom activating property is left. Warm alcoholic extraction of such serum yields, however, a large quantity of lecithin. In the case of non-activating serums no venom activating fats appear in the ethereal extract. Lecithin exists in such serum in no less quantity than in the activating kind.

The addition of oleinic acid or its soluble soaps to a non-activating serum, in a ratio which corresponds to the percentage of fatty acids or soaps contained in some of the easily activating serums, will make the serum highly active in regard to venom.

In normal serum of dog there exists, besides the group of activators already mentioned, another kind of venom activators which has been identified as a lecithin compound acting in the manner of free lecithin.

A very sharp differentiation of the hæmolysis produced by this activator and by the other groups of activators is obtained by means of calcium chloride, which is powerless against lecithin or lecithin compounds, but effective in removing the action of the latter. This lecithin containing proteid can be precipitated by half saturation with ammonium sulphate, but is perfectly soluble in water, and is not coagulated in neutral alkaline salt solutions upon boiling. Alcohol precipitates a proteid-like coagulum and extracts lecithin from it; ether does not extract lecithin from this compound.

Non-activating serums do not contain any such lecithin compound.

Lecithin contained in other serum proteids, mainly as lecithalbumin, and perhaps as contained in globulin, is not able to activate venom. This is true of all the serums with which I worked; it matters not whether these fractions (obtained with ammonium sulphate) belong to the most activating serum (dog) or to the non-activating serum (ox).

The non-coagulable portion of all heated serum contains a venom activator of the nature of lecithin. This activator is contained in a non-coagulable proteid described by Howell which is identical with Chabrié's albumon. As there is no ether-extractable lecithin in this portion of the serum, the activating property of heated serum must be due to this proteid compound of lecithin. That this lecithin proteid does not pre-exist in normal serum but is produced by the action of high temperature is true of all serums except that of the dog. In venom activation we know now that lecithin becomes reactive with venom when it is transformed from other proteid compounds into the non-coagulable form, the albumon. Howell's view of the non-existence of the non-coagulable proteid in normal serum seems to receive a biological support from venom hæmolysis.

Ovovitellin derived from hen's egg is one of the best venom activators of the lecithin proteid type.

The cause of venom susceptibility of various kinds of blood corpuscles does not depend upon the existence of lecithin in the corpuscles, but solely upon the amount of fatty acids, and perhaps, also, soaps and fats, contained in the corpuscles. The protection which calcium chloride gives against venom hæmolysis is proof of the absence of lecithin activation. From the stroma of susceptible corpuscles fatty acids or some fats can be extracted with ether. After ethereal extraction the stroma becomes non-activating, while the extract contains fatty acids and some soaps or fats, which when added to venom-resistant corpuscles render the latter vulnerable to venom. The corpuscular solution of non-activating corpuscles does not contain enough fatty acids. The larger the amount of fatty acids and soaps in the corpuscles, the easier the cells undergo

venom hæmolysis. Lecithin exists in the stroma of all kinds of corpuscles, but in a form unavailable for venom activation.

The somatic cytolytic processes caused by venom requires intracellular complements. The experiments performed on the cells of liver, kidney, testis and brain of the guinea-pig and rat indicate that the substances which act as complements are inactivable by calcium chloride.

ON THE INFLUENCE OF THE REACTION AND OF DESICCATION UPON OPSONINS.[1]

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Whether the opsonic property of normal and immune serums, to which the writings of Wright and Douglas[2] have called renewed attention and endowed with special significance, is a new and hitherto undescribed property of serum or a special function of the usual immune bodies of serum is still a question of dispute.

Bulloch and Atkin,[3] Neufeld and Rimpau,[4] Barratt,[5] Neufeld and Töpfer,[6] Hektoen,[7] and Keith[8] share the view of Wright and Douglas that the opsonic action is due to the presence of certain hitherto unrecognized, distinct bodies, while Savtchenko,[9] Besredka,[10] and Dean[11] are inclined to consider the opsonins as identical with the amboceptors (fixators). Quite recently Muir and Martin[12] brought forth evidence that opsonization depends upon the coöperation of two substances, one of which, at least, closely resembles complement of serum in certain of its biological properties. Still later Levaditi and Inmann[13] asserted that opsonin are nothing but the complements of serum.

It is not my intention to enter into this discussion, but, for my purpose, it is necessary to mention that in common with complement, so-called, the opsonins are absorbed or fixed by sensitized bacteria, blood corpuscles, specific precipitates,[14] and indifferent

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[1] Received for publication April 30, 1907.

[2] Wright and Douglas, *Roy. Soc. Proc.*, 1903, lxxii, 357; 1904, lxxiii, 128.

[3] Bulloch and Atkin, *Roy. Soc. Proc.*, 1905, lxxiv, 379.

[4] Neufeld and Rimpau, *Deut. med. Woch.*, 1904, xxx, 1458.

[5] Barratt, *Roy. Soc. Proc.*, 1905, lxxvi, 524.

[6] Neufeld and Töpfer, *Centralbl. f. Bakt.*, etc., 1905, xxxviii, 456.

[7] Hektoen, *Jour. of Infect. Diseases*, 1906, iii, 434.

[8] Keith, *Roy. Soc. Proc.*, 1906, lxxvii, 573.

[9] Savtchenko, *Ann. d. l'Inst. Pasteur*, 1902, xvi, 106.

[10] Besredka, *Ann. d. l'Inst. Pasteur*, 1904, xviii, 363.

[11] Dean, *Roy. Soc. Proc.*, 1905, lxxvi, 506.

[12] Muir and Martin, *Brit. Med. Jour.*, 1906, Pt. ii, 1783.

[13] Levaditi and Inmann, *Compt. rend. d. l. Soc. d. Biol.*, 1907, lxii, 725.

[14] Muir and Martin, *Brit. Med. Jour.*, 1906, Pt. ii, 1783.

bodies[15] as well as non-sensitized bacteria,[16] and exhibits thermolability and susceptibility to deterioration by age, similar to that which complement exhibits. Besides the liability to undergo adsorption and the high degree of thermolability, no other characteristics of ferments have been ascribed to opsonins. Were opsonins recognized as special ferments certain facts of theoretical and practical import should follow. Among other things we should look for an optimum of activity to be exhibited in the presence of a certain reaction which might or might not be that of the sample of serum which is being studied. If the degree of activity depended upon a given reaction then to obtain the optimum indication, upon which alone a measure of the quantity of opsonin could rest, this reaction would have to be secured. I have, therefore, made a study of the influence of the reaction upon the opsonic power of several blood serums for B. typhosus, B. dysenteriae, Streptococcus, and Staphylococcus aureus.

The results of these experiments harmonize so well that those alone relating to Staphylococcus aureus will be given in this paper.

THE INFLUENCE OF THE REACTION UPON OPSONINS.

The Influence of Alkalinity.—The degree of alkalinity of several serums were first titrated. Lacmoid paper was employed as indicator. The titration was conducted in the following manner. To 1 c.c. of serum $\frac{1}{20}$ N. solution of hydrochloric acid was gradually added until the reaction reached neutral. The amount of acid required for complete neutralization was taken as representing the degree of alkalinity of each serum. The resulting fluid was then made up to 5 c.c. with 0.9 per cent. salt solution. Thus the final mixture had a strength of one fifth of the original serum. By this means it was possible to prepare a series of mixtures, in which 0.5, 0.3, 0.2, 0.1 and null alkalinity were left unneutralized. These fluid mixtures were used for opsonization, and the results are given in Table I. The cross signs under each column represent the average number of the bacteria taken up by a phagocyte, each cross standing for three bacteria.

[15] Simon, Lamar and Bispham, *Jour. of Exper. Med.*, 1906, viii, 651.

[16] Bulloch and Atkin, *Roy. Soc. Proc.*, 1905, lxxiv, 379.

The bacteria were Staphylococcus aureus, twenty-four hours old, and the leucocytes human. The tubes were incubated at 37° C. for thirty minutes, and the technique employed was that given by Wright.

TABLE I.

	Varying Quantities of $\frac{1}{10}$ N. HCl Added to Leave the Indicated Degree of Alkalinity Unneutralized. Total Volume 5 c.c.					
	Original Alkalinity.	Alkalinity Left 0.5 c.c.	Alkalinity Left 0.3 c.c.	Alkalinity Left 0.2 c.c.	Alkalinity Left 0.1 c.c.	Neutral Reaction.
Dog	0.5 c.c. +++	+++	+++	++++	++++	++++
Ox	0.75 ++++	++++	++++	+++++	++++++	++++++
Pig	0.8 +++++	+++++	+++++	++++++	+++++++	+++++++
Rabbit	0.65 +++	+++	+++	++++	++++	++++

As the foregoing table shows, a greater degree of opsonization is obtained at the neutral reaction than at the inherent alkaline reactions of the serums.

For the next series of experiments the inhibitory action of alkalinity upon opsonins was made more apparent by increasing the alkali in an ascending scale.

TABLE II.

	Original Alkalinity.	Amount of $\frac{1}{10}$ N. NaOH Added. Total Volume 5 c.c.				
		0	0.5 c.c.	1 c.c.	2 c.c.	3 c.c.
Dog	0.5 c.c.	+++	++	+	Negative	Negative
Ox	0.75	++++	+++	+	"	"
Pig	0.8	+++++	+++	+	"	"
Rabbit	0.65	+++	++	+	"	"

When 2 c.c. of $\frac{1}{20}$ N. sodium hydrate solution are added to 1 c.c. of a serum the opsonizing property of the latter completely dis-

TABLE III.

Neutralized Serum.	Amount of $\frac{1}{10}$ N. NaOH Added. Total Volume 5 c.c.					
	0	1.2 c.c.	1.4 c.c.	1.6 c.c.	1.7 c.c.	1.8 c.c.
Dog	++++	++	+	±	Negative	Negative
Ox	++++++	++	+	±	"	"
Pig	+++++++	+++	+	±	"	"
Rabbit	++++	++	+	±	"	"

appears; 1 c.c. of this solution added to 1 c.c. of serum reduces the opsonic activity to the minimum. Table III shows the maximum alkalinity in which opsonins are still able to act.

An alkali content approaching 1.6 c.c. suppresses the activity of opsonins. It is rather remakable that this degree of alkalinity is only about twice as high as that possessed by the majority of normal serums.

In Table I. it is shown that the activity of opsonin is greater at the neutral point than at the alkalinity possessed by normal serums, but the difference was not a marked one. In the following experiment the inhibitory influence of the native alkalinity is brought to light by means of dilution. The normal serum of the pig was neutralized with hydrochloric acid and divided into two portions. To one portion sodium hydrate solution was added to make it contain just enough alkali to reproduce the original degree of alkalinity, namely, 0.8 c.c. of $\frac{1}{20}$ N. To the second portion no alkali was added, but it was employed in the neutral reaction. These two portions were used for opsonization in ascending dilutions.

TABLE IV.

Dilution of the Neutralized Serum (Pig).	Alkalinity $\frac{1}{20}$ N. NaOH 0.8 c.c.	Alkalinity $\frac{1}{20}$ N. NaOH 0.8 c c. Neutralized Back with $\frac{1}{20}$ N. HCl 0.8 c.c.	Control in Saline with Neutralized Serum Alone.
1:5	+++++	++++++	++++++
1:7	++++	+++++	+++++
1:10	+++	+++	+++
1:15	+	+++	+++
1:20	±	++	++
1:25	—	++	++
1:30	—	++	++
1:35	—	+	+
1:40	—	+	+
1:50	—	—	—
1:60	—	—	—
1:80	—	—	—

As Table IV. shows, the alkali restrained the opsonic action markedly and the minimum opsonization with the alkalinized portion was between 1 : 15 and 1 : 20, while the neutral portion was still active at 1 : 30 to 1 : 40 dilutions. This restraining effect of alkali was not so evident in the concentrated state of the serum, but was rapidly developed as the dilution was increased. It may

be remarked in passing that the inhibitory influence of the reaction on ferments is by no means a quantitative one. No matter how large a quantity of ferment is present in a mixture no action follows if the reaction is highly unfavorable. When the reaction is merely such as to inhibit partially, only so much effect is obtained as the degree of optimum permits. Under such circumstances, the greater portion of ferment may remain inactive. It is only when ferment finds itself in a fluid of optimum reaction that real quantitative differences can be manifested. The opsonic indices can seldom be driven beyond four, and usually not beyond two or three, in spite of repeated vaccination with bacteria. The reason for this limit is not at once apparent, but it is not improbable that even here the reaction of the serum may play a part. Judging from my experiments, the estimation of the opsonic index should be made at the neutral reaction and in a diluted serum. The advisability of dilution has been pointed out especially by Simon and his co-workers; and with this idea the results of Neufeld and Rimpau with certain immune serums agree.

The Influence of Acidity.—Opsonins having been shown to be more active in a neutral than in an alkaline medium, the next point to be examined was the influence of the acid reaction upon the opsonization. Table V shows that opsonins are highly sensitive to the acid reaction.

TABLE V.

Neutralized Serum.	Amount of $\frac{1}{20}$ N. HCl Added to the Neutralized Serum. Total Volume 5 c.c.					
	0	0.1 c.c.	0.2 c.c.	0.3 c.c.	0.5 c.c.	0.7 c.c.
Dog	++++	+++	++	+	±	Negative
Ox	++++++	+++	++	+	±	"
Pig	++++++++	+++++	+++	+	±	"
Rabbit	++++	+++	++	+	±	"

If to the neutral serum is added 0.5 c.c. of $\frac{1}{20}$ N. hydrochloric acid solution, no opsonic action is to be obtained. The quantity of acid indicated in the table was added to an amount of the neutralized serum corresponding to 1 c.c. of the original.

Are Bacteria Opsonized in the Presence of Unfavorable Reaction? The restoration of opsonic activity.

Bacteria suspended in a serum which contains enough acid or alkali to suppress opsonization under ordinary conditions were centrifugalized and washed in 0.9 per cent. salt solution. The washed bacteria were then mixed with leucocytes and incubated as usual. It was found that these bacteria were not taken up by leucocytes. Thus it can be concluded that when the reaction is unfavorable, opsonins do not attach themselves to the bacteria. An analogous phenomenon was encountered by Hektoen and Ruediger[17] in the case of certain antiopsonic neutral salts.

In these respects, opsonins seem to differ from most bacteriolytic and hæmolytic amboceptors, for they sensitize the corresponding cells in a medium containing an inhibitory amount of acid for opsonins. The sensitiveness of opsonins to slight degrees of acid and alkali is similar to complement, although opsonins surpass complements in sensitiveness.

The fact was ascertained that inactivation of opsonins by means of an unfavorable reaction is a reversible reaction. If the excess of acid or alkali is removed by neutralization the opsonins become active once more. In this respect they resemble complements.[18] A difference is observed in that opsonins are more active in neutral media, and complements more active in alkaline media. Further calcium chloride reduces the action of complements but not of opsonins, provided the concentration of this chemical used does not exceed $\frac{1}{10}$ N.

Organic and inorganic acids in concentration of 1 N. render the serum opsonically inactive even after neutralization. The modification produced is irreversible.

Before leaving this topic, I wish to speak of an incident concerning the effect of small quantities of acids on the opsonic power of serum. At the beginning of this study, I employed $\frac{1}{40}$ N. solution of various acids—hydrochloric, nitric, sulphuric, formic, acetic, propionic, lactic, butyric, oxybutyric, citric, tartaric, oxalic, male-

[17] Hektoen and Ruediger, *Jour. of Infect. Diseases*, 1905, ii, 528.

[18] Noguchi, On the chemical inactivation and regeneration of complement. Read at the annual meeting of the Society of American Bacteriologists, Dec. 28, 1906.

inic, fumaric, itaconic, citraconic and glycerino-phosphoric—and as each in turn was added in small quantities to the serum the opsonic index was observed to rise. Contrary to my expectation, the addition of acid gradually but perceptibly increased the opsonic power of the serum. This first led me to think that acids might themselves act as opsonins. But the power was not further increased as the neutral point was reached, and from that point on, the increasing acidity finally suppressed it altogether.

EFFECT OF DESICCATION AND DRY HEAT ON OPSONINS.

Effect of Desiccation.—Opsonins are highly labile bodies. Their action disappears from the serum when it is allowed to stand for several days. In this respect they resemble complements. But no experiment has been recorded which tells us whether opsonins are obtainable in the dry state or not. I have tested this point. Normal serums of dog, ox and pig were dried at 23° C. with the aid of an air current, and the dissolved masses were tested again for their opsonic power. It was found that the opsonic power of the serum is not noticeably reduced by drying in this way. The next point was whether the opsonins would endure longer in the dried state. To determine this, I employed three samples of dried serum which had been preserved in the laboratory for two years. The serums were obtained from the rattlesnake, horse and ox, and were dried at a temperature approaching 48° C. Although I am unable to state the original activity at the time of drying, I can state that all three serums exhibited marked opsonic powers. Thus opsonins, once they are dried, are not labile substances.

On the other hand, dehydration of serum by means of alcohol renders the opsonins completely inactive. This fact would show that opsonins differ at least in this respect from many ferments, which stand treatment with alcohol, if not too prolonged.

Effect of Dry Heat.—The normal serum is robbed of its opsonic power by temperatures ranging from 55° to 60° C. Even immune serums, which are the most resistant in this respect, lost the greater part of their opsonic power at these temperatures, although traces may persist in a serum heated to 65° C. On the whole, opsonins are more thermostabile than complements, although less than the

usual immune bodies. The slight difference in the thermal resistance obtained by different investigators may depend upon differences in the reaction or some other physical or chemical conditions under which the tests were made.

Opsonins resemble ferments in their behavior to high temperature in the dry state. I have subjected the dried serums of ox, rabbit and dog to the temperature of 100°, 120°, 135° and 150° C., and then tested them for their opsonizing power. When the serums are heated to 120° C. and above, they become almost insoluble in water. To examine their activity, they must be powdered and emulsified in water. The bacterial emulsions were added to the former emulsions and the whole incubated at 37° C. for two hours. The result is given in Table VI.

TABLE VI.

	Opsonic Activity of the Heated or Unheated Dried Serum.					
	Unheated Dried Serum.	Dried Serum Heated to 100° C.	Dried Serum Heated to 120° C.	Dried Serum Heated to 135° C.	Dried Serum Heated to 150° C.	Unheated Dried Serum, Redissolved and then Heated to 56° C.
Ox serum	Active phagocytosis	Active phagocytosis	Active phagocytosis	Less active, but positive.	Very irregular occurrence of phagocytosis	Negative.
Rabbit serum (vaccinated)	"	"	"	"	"	"
Dog serum	"	"	"	"	"	"

From the foregoing experiment a high degree of resistance of the dried opsonins to high temperatures may be inferred. It is important to note that the dried serums regain their thermolability when redissolved in water, as is shown in the last column of the table.

Hence, it would appear that opsonins possess in common with ferments the property to resist in the dry state the action of high temperatures.

In the course of the experiments on desiccation and high temperature on opsonins, I took the opportunity of examining complements in the same manner.

It developed that complements do not disappear from the serum when it is dried at 23° C. The desiccated serum retained for several months complements unaltered in quantity and in activity.

. Heating the dried serum to 100°, 120° and 135° C. does not deprive it of complementary action.

SUMMARY.

Opsonins reveal their maximum action in a medium of neutral reaction. No opsonization takes place in a serum which contains an amount of alkali corresponding to more than 1.6 c.c. of a $\frac{1}{20}$ N. solution, or acid more than 0.5 c.c. of this concentration per 1 c.c. of serum. Of several normal blood serums titrated (lacmoid used as indicator) the average alkalinity was found to be equivalent to about 0.8 c.c. of $\frac{1}{20}$ N. solution.

The opsonic index obtained in the native serums is not the expression of the action of the whole content of opsonins, but only so much as the degree of optimum of the reaction permits to come into action. Estimation of the opsonic power should, therefore, be made in a medium of neutral reaction and in diluted serum.

All serums have their opsonic power increased by diminishing the native alkalinity.

Opsonins whose activity is suspended by an unfavorable reaction become immediately active as soon as the reaction is brought back to the neutral point, unless the acid or alkali employed approaches the strength of 1 N., at which point the alteration becomes permanent.

Treatment of a serum with alcohol robs it of its opsonic power. The opsonic power of serum remains unaltered upon desiccation at 23° C. In the dry state opsonins are preserved for two years.

The temperatures of 100°, 120°, 135° and 150° C. do not destroy opsonins of the dried serum.

Complements of serum are also siccostabile and are preservable in that state for several months. Dry heat of 135° C. reduces but does not destroy the complementary power of the dried serum.

The opsonins and complements of the dried serum regain their original thermolability when they are dissolved in a proper amount of water.

Lastly, it may be emphasized that opsonins exhibit in their sensitiveness to reaction and resistance in the dry state to high temperatures certain properties characteristic of the ferments.

SPIROCHÆTA (TREPONEMA) PALLIDA AND SYPHILIS.

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PLATE XVII.

The extensive bibliography on experimental syphilis and spirochæta pallida has been brought together and reviewed recently by Hoffmann,[1] Neisser,[2] R. Kraus,[3] Glass,[4] and Buschke and Fischer,[5] in whose publications the present state of our knowledge on these subjects can be readily found. The weight of opinion is strongly in favor of accepting the transmissibility of the syphilitic virus to the lower and the higher monkeys, and to the eyes of rabbits, guinea-pigs[6] and dogs.[7] The almost constant demonstration of spirochæta pallida in syphilitic products and in the organs of fœtuses and infants inheriting active forms of syphilis, and the close association of the spirochætæ with the specific lesions, has led the majority of workers in this field to look upon the pallida as the probable cause of syphilis. The criticisms which have been made of this view have, in the main, not weakened it seriously, and must, in the nature of things, remain as criticisms until such time as the pallida is obtained in pure cultivations with which inoculation tests can be carried out. Apparently we are no nearer the cultivation of the pallida than we were immediately after its discovery. The criticism that has, in the past, aroused the largest degree of interest holds that the tissue spirals, exhibited by silver impregna-

[1] Hoffmann, Die Ätiologie der Syphilis nach dem gegenwärtigen Stand unserer Kenntnisse, Berlin, 1907.

[2] Neisser, Die experimentelle Syphilisforschung nach ihrem gegenwärtigen Stande, Berlin, 1906.

[3] Kraus, Handbuch der Hautkrankeiten, 1905, p. 318.

[4] Glass, Inaug.-Diss., Leipzig, 1906.

[5] Buschke and Fisher, *Arch. f. Dermatol. u. Syphilis*, 1906. lxxxii, 63.

[6] Bertarelli, *Cent. f. Bakt.*, Orig., 1907, xliii, 448.

[7] Hoffmann and Brüning, *Deut. med. Woch.*, 1907, xxxiii, 553.

tion, are not micro-organisms, but histological structures such as nerve filaments, cement lines, elastic fibrils, etc. Saling and W. Schultze[8] especially have championed this view which has found some adherents. The force of it has undoubtedly been considerably enhanced by the fact that a method for staining the pallida in tissues by means of aniline dyes has not been found, and, at the time of its promulgation, a method for silver staining of the pallida in film preparations had also not been found. It was possible to stain the pallida quite constantly in film preparations with Giemsa's stain, with Proca's stain and with many other special aniline stains; but often there was observed a striking disproportion between the large numbers of spirals of pallida form impregnated in tissues with silver and the smaller number demonstrable with anilines in the films. Differences in size and thickness of the spirals have also been noted and described, depending upon whether they were seen in film preparations stained with anilines when they appeared thin and delicate, or in tissues impregnated with silver when they appeared thicker and coarser. All these disagreements have been used to discredit the micro-organismal nature of the spirals and the identity of the film and the tissue spirals. The discussion provoked by Saling and Schultze's criticism has been wholesome in bringing out rapidly new facts relating to the pallida that would probably have come out finally, and which tend to strengthen the view of its ætiological position in syphilis. Mühlens[9] has undertaken to give a categorical refutation of Saling and Schultze's claims.

My interest in spirochæta pallida began soon after its description by Schaudinn and Hoffmann and has continued until the present time. I have not worked continuously with the pallida in relation to syphilis during the intervening period, but I have taken pains to collect experience regarding the subject. Through the courtesy of the staff of the City Hospital and of Dr. Ryder of the Sloane Maternity Hospital, I have had numerous opportunities to study cases of acquired and congenital syphilis and other venereal diseases.

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[8] Saling, *Cent. f. Bakt.*, Orig., 1906, xli, 712, 812; 1906, xlii, 38, 120. Schultze, *Berl. klin. Woch.*, 1906, xliii, 1213; 1906, xliii, 1654.

[9] Mühlens, *Cent. f. Bakt.*, Orig., 1907, xliii, 586, 674.

I have collected notes on the occurrence of the pallida in a wide variety of syphilitic lesions and on the relation of film and tissue spirals which are essentially in accord with the experiences of others who have worked with a large and varied material. The discrepancies in numbers met with, I have endeavored to remove in the case of films, by employing the Proca staining method without the alcohol fixation, with a view to enhancing the action of the mordant and increasing the certainty of the staining result. This modification is an advantage in certain instances, since by means of it more uniform staining of the pallida could be obtained than with the original method or with Giemsa's stain in any of its several ways of application. But my experience taught me that other factors beside the aniline stain, or the particular manner of its application, affected the result. Just what these factors are, I cannot say, but they affect either the pallida itself or the medium in which it lies. The conclusion I reached is that silver-impregnation often exhibits many more spirals than the aniline dyes because it is effective under conditions which interfered with the anilines' giving a differential result. I experimented somewhat with the direct silver staining of spirochætæ in film preparations without success, until Stern[10] published his method which is very simple and very effective and has already removed many of the discrepancies which I had previously observed. With this introduction, I propose to record a few of my notes, since they bear upon certain contested points in the ætiology of syphilis.

Appearance and Persistence of Spirochæta pallida in Secondary Lesions.—The patient, a young woman, was exposed to venereal infection in November, 1906, and noticed the first lesions, consisting of condylomata and skin maculæ in February, 1907. I first saw the patient early in March, at which time the condylomata had nearly disappeared, but the maculæ were distinct over the back and chest. On March second, there appeared on the lower lip, near the left angle of the mouth, an elevated flat papule of pale grey color, covered with intact epithelium. The epithelial layer was lightly scraped away with a curette and impression and smear films made from the exuded serum. The films stained by the modified Proca and by the Giemsa method gave very many typical S. pallida. The patient was at this time receiving deep injections of mercurial salts. The papule increased in size in the next days, and on the twelfth instant, a second examination was made with similar results. On the twentieth

[10] Stern, *Berl. klin. Woch.*, 1907, xliv, 400.

instant, the papule was stationary and the examination gave many typical pallida. On April 12, this papule had begun to regress and a second papule, smaller in size, made its appearance on the lower lip near the opposite angle of the mouth. Films made from both papules on this date showed fewer pallida in the first lesion than on the previous examinations, and pallida in the second lesion but fewer in number than in the first papule. These papules were examined at intervals of one or two weeks, until they gradually disappeared. The second one to develop was never as rich as the first in pallida; and the first papule continued to show pallida in diminishing numbers until its final disappearance about June 1. During this period of examination, other papules appeared on the tongue and pharynx and they showed the pallida on examination. While these lesions were developing or remaining stationary, the skin maculæ were disappearing.

The films from the papules of the lip never showed any micro-organism except S. pallida, while those from the tongue and pharynx, with which the mouth secretions became admixed, showed other micro-organisms including the mouth spirals. It is worth mentioning that evidences of degeneration or dissolution were not found in the pallida obtained from the disappearing lesions of the lip. During the stationary period, the number of pallida was large and the forms distinct. The number of the pallida decreased very slowly, even when regression of the lesion set in, but the forms remained distinct. Fragmented spirals were never observed.

A large number of films were prepared at the different examinations and stored in the refrigerator at 2°–4°C. They retained their staining properties unimpaired for many months. Subsequently, some of these were stained directly with silver nitrate which brought out the spirals with great distinctness. The number exhibited was about the same as was shown by the Proca stain and depended somewhat upon the length of immersion in the silver bath. The silver is precipitated slowly upon the spirals, and hence a deeper and more complete impregnation is secured after an exposure of from three to five days than after a shorter exposure. When the impregnation is less perfect, the spirals appear broken into a series of comma-like figures, or they are indicated by a sinuous line of dots.[11] Longer immersion usually brings out the unbroken spirals in strong relief. The precipitation in the blood corpuscles and serum is so fine as to give merely a faintly colored ground against which the intensely black pallida stand out sharply. Disturbing coarse precipitates do not occur. Heavier films can be employed for the silver impregnation than for many of the anilines.[12]

This case is of interest in showing the close and immediate connection of spirochæta pallida with the developing and persisting

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[11] Stern, *op. cit.*

[12] The length of the immersion in the silver bath will be determined by the strength of light to which the immersed films are exposed. It is, I think, better to employ very weak and diffuse light and not strong diffuse light, so as to bring the silver down slowly and to avoid deep coloration and objectionable precipitations. The direction of Stern to wait until a metallic film appears is useful, but this film often appears before the impregnation is complete. Excessive impregnation causes the spirals to appear coarser and of uneven contour.

secondary lesions of syphilis, and the gradual disappearance of the pallida, without exhibiting direct evidences of dissolution, with the regression of the lesions. The facts observed are interesting for another reason, namely, that the number of the pallida brought out on the films was about equal by the mordanting Proca stain, by the Giemsa stain (immersion for one or two days), and by the silver impregnation method.

Apparent Discrepancy in the Finding of S. pallida upon the Surface and in the Interior of Lesions.—The patient was a young woman who presented many typical flat condylomata about the vulva and anus. Film preparations from a superficial scraping of a condyloma stained by Proca's and Giemsa's method showed many spirochætæ of the pallida type, some of the refringens type and other micro-organisms. Three days after the examination, the treatment in the interim having consisted of calomel powder, a condyloma was excised. Impression and smear preparations from the interior of the lesion were stained by the methods used for the superficial scrapings, but no spiral or other micro-organisms were shown. A very careful and laborious search was made of many films without finding any pallida. After the silver impregnation had been used successfully on films from other cases, it was applied to the films, some of which had been preserved in the refrigerator, prepared from the interior of the condyloma. All the films impregnated with silver showed typical pallida type of spirals.

The second patient was a man who presented himself some weeks after the appearance of an ulcerated lesion of the glans penis. A typical skin eruption and general adenitis existed. Circumcision was performed, and when I examined the patient, the ulcer was clean and indurated. Films were made from the lymph exuded after light curetting. No spiral organisms could be found upon staining with the Proca and the Giemsa methods. Silver impregnation gave a small number of typical spirals of the pallida type.

A discrepancy has been noted repeatedly in the number of pallida shown in films and the far greater number shown in tissues impregnated successfully with silver, and certain discrepancies have also been noted in the clinical appearances, suggesting unmistakable syphilitic infection and the successful demonstration of the pallida in the lesions. It is true that with the development of greater facility in examination, fewer failures in these examinations have to be recorded; but it is also true that the number of pallida demonstrable in the films may be very small. It would appear as if the medium in which the pallida at times finds itself may affect the staining result considerably. I am inclined to regard the medium as influencing the staining in certain instances rather than a peculiar

condition of pallida itself. Apparently the unfavorable condition of medium is less effective against the silver impregnation, and hence I am disposed to think that by employing it systematically in this class of examinations fewer discrepancies will have to be recorded in the future. A very striking example of the great value of the silver impregnation of the films is supplied by an examination of a macerated fœtus, the facts of which follow.

Congenital Syphilis with Colony-like Masses of S. pallida.—The mother had three previous pregnancies. The first was normal, the child dying at three months of age; the second was a miscarriage at the third month; the third child was still born. Two years ago, she had a general rash followed by pharyngitis and alopecia and ulcers of legs. Scars of the last still remain. The mother felt signs of life in her present pregnancy until two weeks before entering hospital. During the two days she was in hospital before the miscarriage, no signs were discovered. Miscarriage at about the seventh month. Moderate degree of maceration of fœtus; no decomposition. The epidermis had come way over a large part of the surface of the body exposing a pinkish cutis showing punctate hæmorrhages. The peritoneal cavity contained much dark, blood-stained fluid. The organs were moderately macerated. Films were made from the skin surfaces, the lungs, liver and adrenal glands and were stained by Proca's method and impregnated with silver.

The Proca-stained films show a small number of pallida in the skin and adrenal gland; while the corresponding films impregnated with silver show large numbers of pallida. The microphotograph (Plate XVII, Fig. 1) shows a colony-like mass and outlying smaller groups of the pallida present in a silver-impregnated film from the skin magnified one thousand diameters, and the microphotograph (Plate XVII, Fig. 2) shows a number of isolated pallida of the same film magnified two thousand diameters. Evidence of transverse division is seen among the spirochætæ.

The films prepared from this fœtus show clearly the great discrepancy which may occur between the results obtained by aniline staining and silver impregnation of the pallida. There can be no doubt that the masses of pallida brought out in the films by the silver-impregnation agree better with the appearances presented by silver-impregnated tissues than the smaller numbers shown by the aniline dye. That the pallida grows sometimes in large felted, colony-like masses on the cutis is proven by this observation. Whether equal growth ever takes place in the living tissues, or whether it is only in the dead fœtal tissues retained in utero that such development takes place, is an open question. Doubtless fœtal tissues are very favorable to the multiplication of the pallida, and

it is easily conceived that the dead, sterile tissues of the fœtus maintained at body temperature, might form a suitable soil for unrestricted growth of the spirochætæ. Many of the changes taking place in the tissues of the macerated fœtus are the result of autolysis; but the products of this autolysis are without active dissolving effect on the pallida and they would appear, in view of the possibility of post-mortem development of the pallida just suggested, not to restrain effectively its growth. I have evidence (see below) that the pallida is far less subject to the disintegrative influences of autolytic tissue ferments than the body cells. On the other hand, I have studied the tissues from a congenitally syphilitic child, dying on the 12th day after birth, in which such a rich development of the pallida had taken place in the lungs and liver (perhaps elsewhere also) as to recall the masses seen in the film from the skin just described. Since so much stress had been laid by some critics of the pallida in relation to syphilis upon an apparent discrepancy in the number of the pallida occurring in macerated fœtuses and in syphilitic infants born alive, I shall describe briefly this instance and two or three others bearing on the latter point.

Spirochæta pallida in Congenitally Syphilitic Infants.—Case 1. Infant died on the twelfth day after birth from repeated and uncontrollable hæmorrhages from the umbilical cord and the gastro-intestinal mucosa. Autopsy performed twenty-four hours after death. The histological examination showed syphilitic pneumonia, and interstitial hepatitis, splenitis, pancreatitis and nephritis. The lung and liver were impregnated with silver nitrate by the original Levaditi method. The lung sections show countless myriads of the pallida in the interstitial tissue and in the alveoli containing the desquamated epithelium, and surrounding and penetrating within the lumen of the bronchi. Many, but fewer, pallida are present in the liver sections within and between the liver cells and in the interstitial tissue.

Case 2. Infant lived one day. Autopsy twenty-four hours after death. Anatomical diagnosis: white pneumonia; syphilitic perisplenitis, hepatitis and nephritis. Sections of the lungs and liver show many typical spirochætæ pallidæ.

Case 3.[13] Premature infant; lived three-quarters of an hour. Mother syphilitic. Autopsy four hours post mortem. Pallida numerous in the skin, in small numbers in the liver and the bile (Proca stain).

Case 4.[14] Mother contracted syphilis seven years before present pregnancy; no symptoms at present. The living child shows a roseolus and papular rash over legs, trunk, etc. A few drops of serum and blood were expressed from an incised macule of the foot; they were rich in spirochætæ.

[13] Flexner, *Medical News*, 1905, lxxxvii, 1106.

[14] *Idem.*

Case 5. Slightly macerated fœtus of sixth month. Mother entered hospital in the sixth month of pregnancy on account of condylomata and œdema of vulva. Examination of a condyloma (Proca and Giemsa stain) gave many pallida. Abortion: pallida in the fœtus.

The next example (Case 6) it that of a slightly macerated fœtus of the eighth month showing extensive areas of white pneumonia (adhesions existed between the pneumonic areas and the chest wall). The pneumonic areas showed large numbers of the pallida, while the adjacent non-pneumonic lung tissue showed few or no pallida (Proca and Giemsa staining). Silver impregnation showed large numbers of the pallida in the infiltrated but not in the non-infiltrated lung tissue. Silver-impregnation of films prepared four months previously and kept in the refrigerator, showed many pallida. The impregnation was less heavy than with other and more recent films, and no striking disproportion in numbers of the spirochætæ existed between the aniline-stained and silver-impregnated specimens. The mother of the fœtus showed no signs of active syphilis, except a small ulcer of the tongue, three or four millimeters in extent, from the depth of which S. pallida was obtained.

Fragments of the lung tissue were kept in the refrigerator (2° to 4.5° C.) for three months. At these temperatures, a slow autolysis without putrefaction goes forward in the tissues. At the end of one month, the tissues were much softened and disintegrated, but the pallida were little, if at all, altered in form and staining properties. At the expiration of three months, the tissues were still further softened and disorganized (no putrefaction) and no pallida could be stained. This observation bears upon the resistance displayed by the pallida to the destructive action of the autolytic ferments.

Our knowledge of the viability of the pallida outside the body is very imperfect. I am, for this reason, led to record the following instance in which the virus was able to produce infection in a macac species of monkey after the excised chancre had been kept in the refrigerator for twenty-four hours.

Viability of S. pallida and Recurrent Syphilitic Lesion in the Monkey.—A chancre of the vulva was excised December 31, 1905, under cocaine anæsthesia. Films showed a moderate number of pallida. Accidental circumstances required the tissue to be kept in the refrigerator until the next day before inoculation of a monkey could be carried out. The inoculation was made into the right eyebrow. The scarifications healed in three or four days. Twenty-four days after inoculation, a small, elevated induration appeared at the site of inoculation, which gradually increased and extended until it reached 2.5 cm. in extent. Scales and later crusts formed over the surface which on removal showed an ulcerated area. A small portion of the node was excised for examination. The his-

tology was that of experimental chancre in the monkey. A slow and gradual recession of the lesion occurred, until at the tenth week, only a small indurated area remained. After a brief quiescent period, a new growth began, which, at the end of the twelfth week equalled the original tumefaction. After a second stationary period, the growth began again to recede about the sixteenth week, and finally quite disappeared. The histology of the recurrent lesion was similar to the original.

I shall not comment further on these notes which are presented as evidence of the relationship of spirochæta pallida to acquired and congenital syphilis.

ADDENDUM.

Since the above paper has been in the hands of the printer, Schmorl (*Deut. med. Woch.*, 1907, XXXIII, 876) has succeeded in devising a method for the demonstration of spirochæta pallida. in the tissues of congenitally syphilitic infants, stained by Giemsa's methods. This demonstration removes another of the objections urged against the microörganismal nature of the spirals and correlates still further the appearances observed in the films and in the tissues.

PLATE XVII.

FIG. 1. Spirochæta pallida impregnated with silver. Film prepared from the skin of a macerated, congenitally syphilitic fœtus. Magnification, 1,000 diameters.

FIG. 2. From the same film as Fig. 1. Magnification 2,000 diameters.

FIG. 1.

FIG. 2.

THE EFFECT OF CERTAIN SURGICAL ANTISEPTICS AND THERAPEUTIC AGENTS ON PHAGOCYTOSIS. I. CARBOLIC ACID, MERCURIC CHLORIDE, BORIC ACID, QUININE HYDROCHLORIDE.[1]

By WILFRED H. MANWARING and HAROLD O. RUH.

(*From the Pathological Laboratory of Indiana University.*)

The present wide interest in opsonotherapy and belief in the importance of phagocytosis in immunity, make it desirable to determine the effect of the commoner medicinal agents on phagocytic power. The effect of the three most important surgical antiseptics, namely, mercuric chloride, carbolic acid and boric acid, and of the most widely used internal remedy in acute infections, quinine hydrochloride, have thus far been determined, and are here presented.

Material and Technique.—Except in the one experiment hereinafter specially mentioned, the material used in this study was defibrinated human blood and streptococci suspended in physiological saline (0.85 per cent. sodium chloride). The streptococci were in all cases obtained from twenty-four to forty-eight hour cultures on blood serum (goat), sterilized at 100° C.

To test the effect of a chemical substance on phagocytosis, increasing amounts of a solution of that substance (whenever possible equimolecular with 0.85 per cent. sodium chloride) were added to a number of test-tubes, and the volumes in the tubes made constant (1 c.c.) with physiological saline. There was then added to each tube a constant amount (1 c.c.) of freshly drawn, defibrinated blood and, at stated intervals, a constant volume (1 c.c.) of streptococcus suspension.

Each tube was immediately immersed in a thermostatic water bath at 37.5° C., and fifteen, thirty and sixty minutes later, smears

[1] Presented before the Chicago Pathological Society, April 11, 1907, and before the American Association of Pathologists and Bacteriologists, at Washington, D. C., May 8, 1907. The work was aided by the Rockefeller Institute for Medical Research. Received for publication June 6, 1907.

were made from it. These smears were stained by Wright's method and the number of bacteria in sixty polymorphonuclear leucocytes in each smear counted.

Experimental Error.—In beginning quantitative work in any field of biological chemistry, it is necessary, first of all, to determine the accuracy of the proposed experimental method—the limits of the experimental error. Without such determination, deductions from experimental data are comparatively valueless. An experiment was therefore undertaken to determine the range of error in work by the above technique.

To do this, ten duplicate tubes were prepared, each containing a mixture of equal parts (1 c.c.) of physiological saline, defibrinated human blood and streptococcus suspension. Smears were made from these tubes and counted, exactly as in the proposed investigation.[2] The data from this experiment are given in Table I.

TABLE I.

Duplicate Bacterial Counts.

		1	2	3	4	5	6	7	8	9	10	Average.
15 Minute Counts	Wright's Index	5.63	5.58	6.18	5.23	5.87	5.83	5.72	5.27	5.53	5.21	5.61
	Error	+ .02	— .03	+ .57	— .38	+ .26	+ .22	+ .11	— .34	— .08	— .40	± .24
	Percentage Error	+ 0.4	—0.5	+10.0	—6.8	+4.6	+3.9	+2.0	—6.1	—1.4	—7.1	±4.3
30 Minute Counts	Wright's Index	[9.63]	8.40	7.65	7.48	7.38	7.35	7.88	8.15	7.83	7.50	7.73
	Error	[+1.90]	+ .67	— .08	— .25	— .35	— .38	+ .15	+ .42	+ .10	— .23	± .29
	Percentage Error	[+24.6]	+8.7	—1.0	—3.2	—4.5	—4.9	+1.9	+5.4	+1.3	—3.0	±3.8
60 Minute Counts	Wright's Index	12.60	11.80	12.15	12.05	11.93	11.35	11.48	10.87	11.03	11.08	11.64
	Error	+ .96	+ .24	+ .51	+ .41	+ .29	— .29	— .16	— .77	— .61	— .56	± .4
	Percentage Error	+ 8.2	+2.1	+4.4	+3.5	+2.5	—2.5	—1.4	—6.6	—5.2	—4.8	±4.1

From this table it is seen that the average error in the fifteen minute counts is 4.3 per cent., with a maximum error of 10 per cent. In the thirty minute counts, with the exception of a single smear, the average error is 3.8 per cent., and the maximum 8.7 per

[2] This experiment, of course, was not attempted till the technique had been mastered by preliminary work.

cent., the one exceptional smear giving an error of 24.6 per cent. In the sixty minute counts, the average error is 4.1 per cent., the maximum 8.2 per cent.

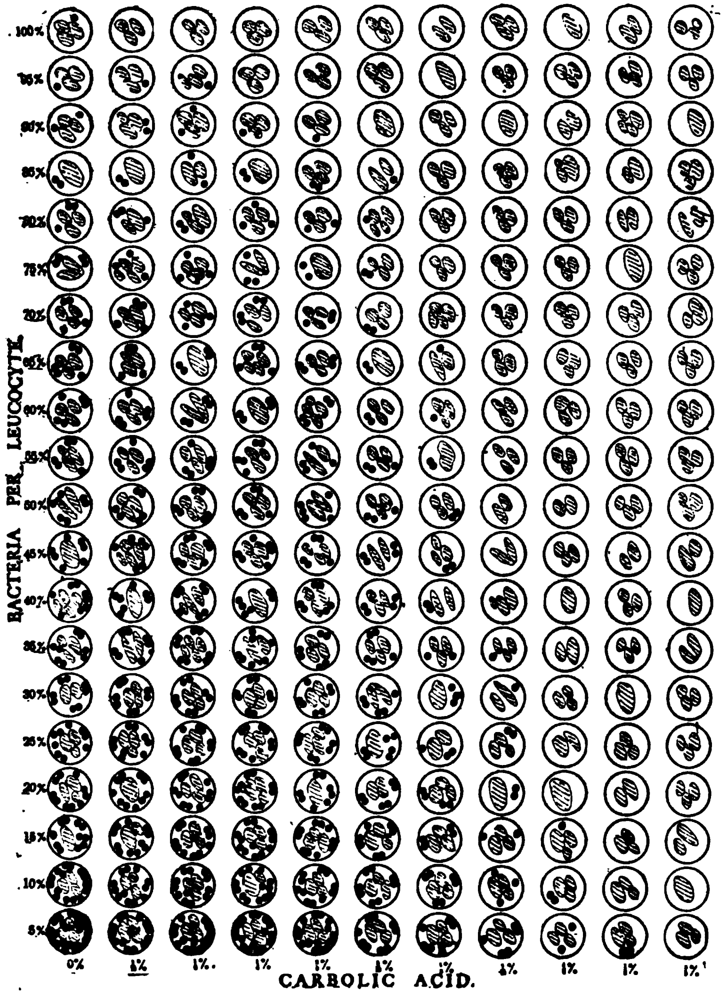


FIG. 1. Influence of carbolic acid on phagocytosis. Human leucocytes; average of 15, 30 and 60 minute counts.

In interpreting data obtained by the above technique, therefore, allowance must be made for a usual maximum error of about 10 per cent., but an occasional error (one count in thirty) of as much

as 25 per cent. must be expected. To eliminate this large experimental error, the average of a large number of data must be used.

Graphic Representation.—In recording data in most fields of quantitative chemistry, it is desirable to select a graphic method of representation. Such a method for the phenomenon of phagocytosis, must give not only the average number of the bacteria per leucocyte, and the number of leucocytes ingesting bacteria, but the numerical distribution of the bacteria among the different leucocytes, as well.

The graphic method selected is shown in the column "0%" of Fig. 1. This column consists of twenty diagrammatic leucocytes,

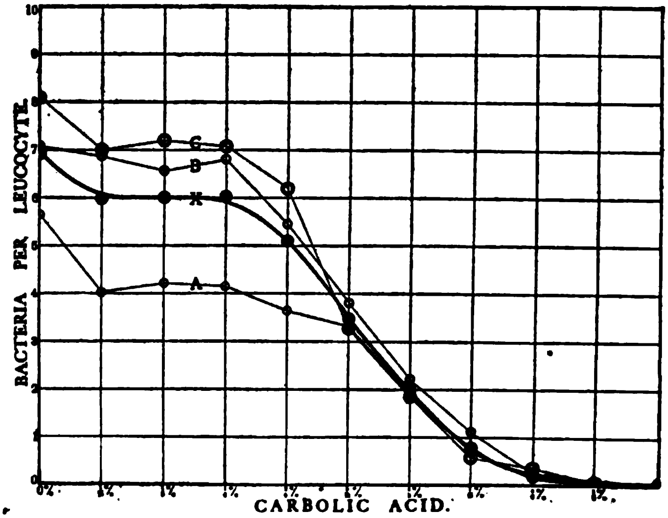


FIG. 2. Influence of carbolic acid on Wright's index. Data from Figure 1, $A = 15$-minute counts; $B = 30$-minute counts; $C = 60$-minute counts; $X =$ average.

each leucocyte representing 5 per cent. of the polymorphonuclear neutrophiles present in a given smear. In each leucocyte is marked the average number of bacteria found in its corresponding 5 per cent.

Carbolic Acid.—The average of the three counts obtained by the above technique, with carbolic acid, gives the data recorded in Fig. 1. These data can be translated in terms of the currently used

Wright's index, by calculating the average number of bacteria taken up per leucocyte. Such calculations are shown graphically by the broken lines *A, B* and *C* of Fig. 2. The average of these observa-

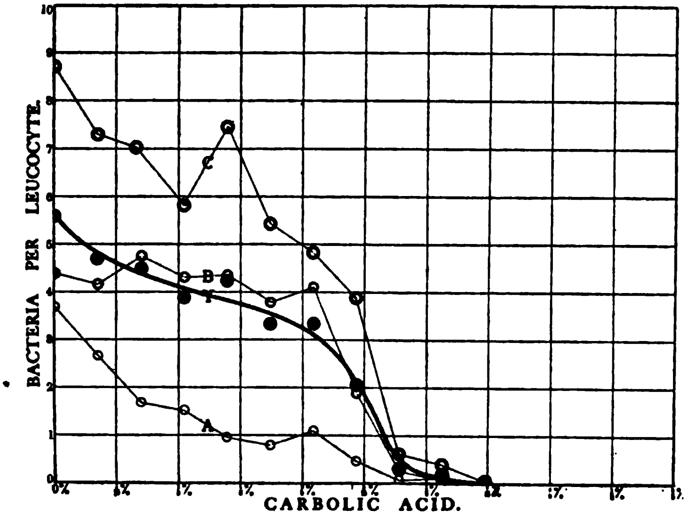


FIG. 3. Influence of carbolic acid on Wright's index. Rabbit leucocytes. *A, B* and *C* = 15-minute, 30-minute and 60-minute counts; *X* = average.

tions, plotted as smooth curves to eliminate experimental errors, as is customary in physical experimentation, is given in the heavy

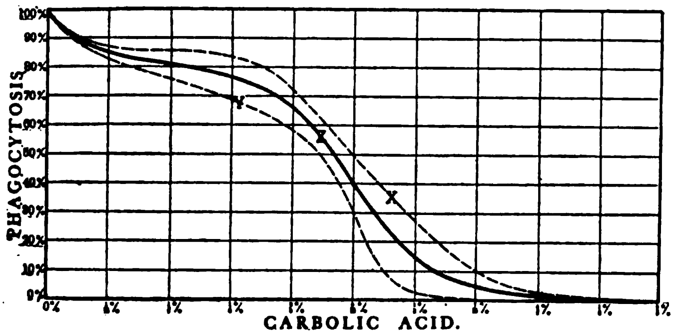


FIG. 4. Influence of carbolic acid on Wright's index; Curves *X* and *Y*, of Figs. 2 and 3, plotted to the same scale. *Z* = average.

curve *X*. A similar curve, obtained with rabbit blood in place of human blood, is given in Fig. 3. The curves of these two experi-

ments, reduced to percentage curves, by taking the phagocytosis in the control tubes of each experiment as 100 per cent., are shown in Fig. 4.

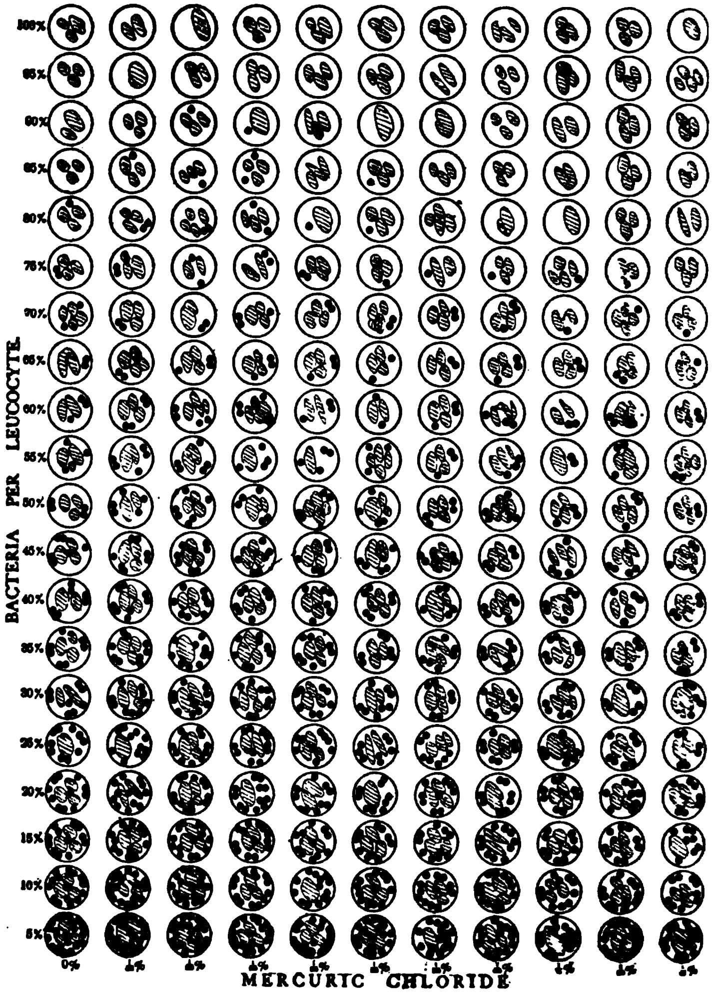


FIG. 5. Influence of mercuric chloride on phagocytosis. Human leucocytes; average of 15- and 30-minute counts.

From this figure, it is seen that the addition of carbolic acid to the experimental tubes causes, from the first, a decrease in phagocytic power, phagocytosis falling off about one third by the time the

carbolic acid reaches a concentration[8] of 2/9 per cent. A further increase in carbolic acid causes an almost precipitous fall in phagocytic power, phagocytosis being reduced seven eighths by the time

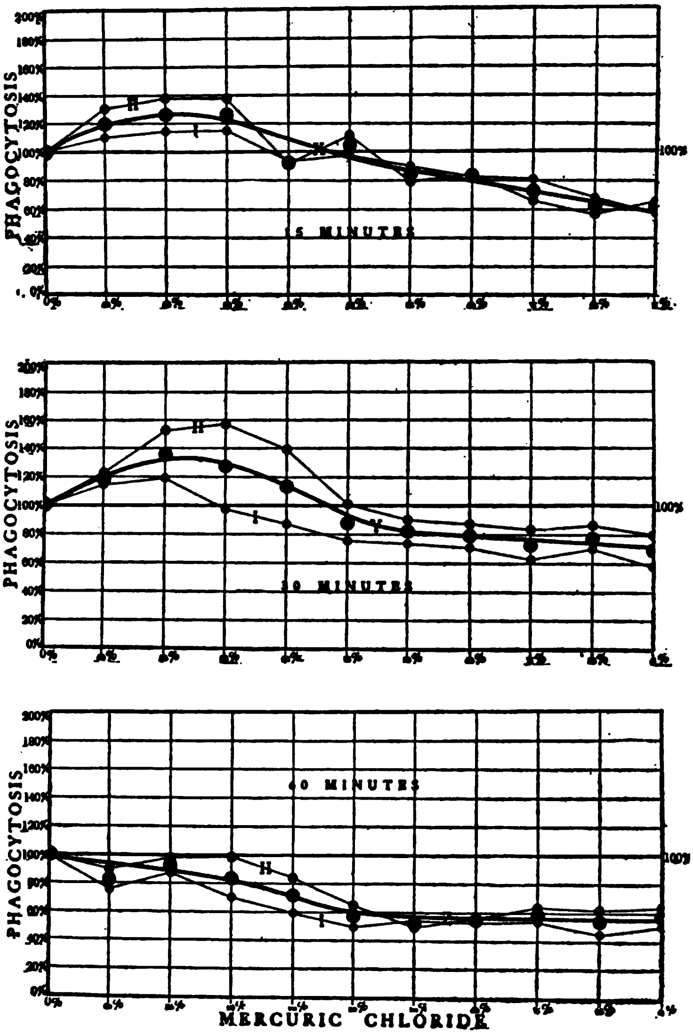


FIG. 6. Influence of mercuric chloride on Wright's index. 15, 30- and 60-minute data plotted separately. I = data from first experiment; II = data from second experiment; X, Y and Z = averages.

[8] Concentration measured in terms of final dilution.

the concentration reaches one third per cent., and entirely ceasing at one half per cent.

Mercuric Chloride.—The average of the fifteen and thirty minute counts, in two experiments with mercuric chloride are given in Fig. 5. Since the sixty minute counts and the earlier counts in these experiments show different effects of mercuric chloride, the influence of this antiseptic on phagocytosis can not be represented by a single curve, as was done with carbolic acid. Three percentage curves were therefore plotted, showing the changes in Wright's index, at the end of fifteen, thirty and sixty minutes. These curves are shown in Fig. 6. In each curve 100 per cent. stands for the

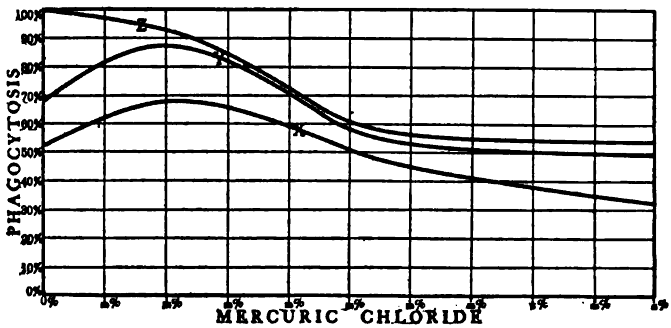


FIG. 7. Influence of mercuric chloride on Wright's index. Curves of Fig. 6 plotted to the same scale.

phagocytosis in the control tubes, containing no antiseptic.

These three curves may be reduced to the same scale by taking the normal sixty minute phagocytosis as 100 per cent. Thus reduced, they are shown in Fig. 7. From this figure, it is seen that mercuric chloride causes, in small amounts, a preliminary stimulation in phagocytosis, followed by a depression. The maximum preliminary stimulation is obtained with a bichloride concentration of 1/300 per cent. As the concentration increases beyond this amount, the preliminary stimulation decreases, and completely disappears at 1/120 per cent.

A further increase in mercuric chloride causes a pronounced fall in phagocytic power, early phagocytosis being reduced 50 per cent. by the time the concentration reaches 1/60 per cent. With con-

centration of 1/200 per cent. and over, phagocytosis practically ceases at the end of thirty minutes.

Boric Acid.—The average of the fifteen and thirty minute counts

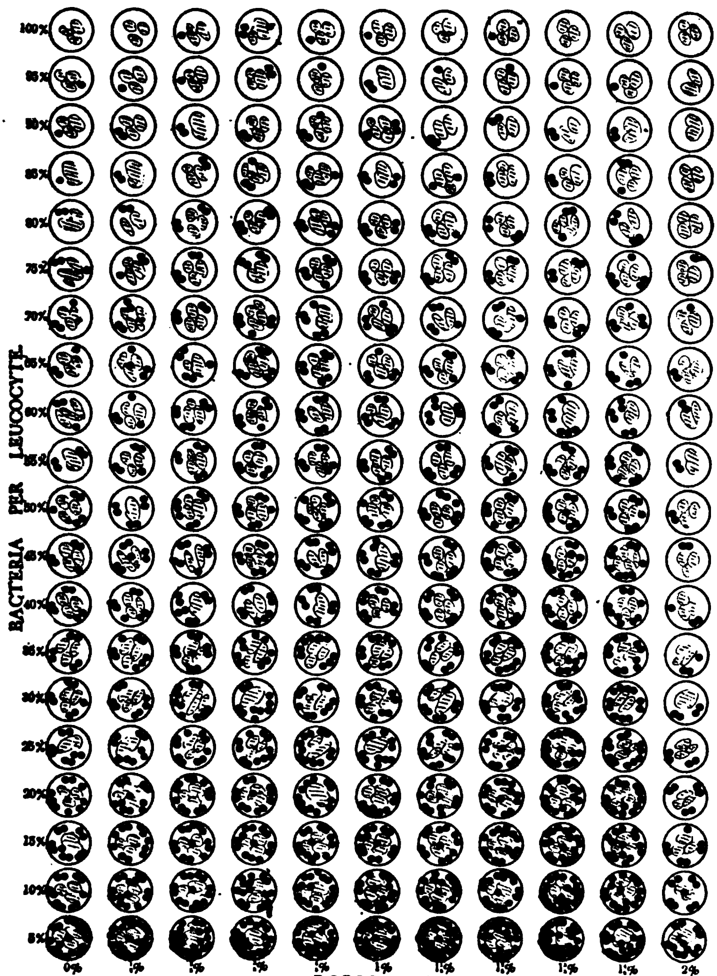


FIG. 8. Influence of boric acid on phagocytes. Human leucocytes, average of 15- and 30-minute counts.

in two experiments with boric acid are given in Fig. 8. With boric acid, as with mercuric chloride, the sixty minute counts

and the earlier counts show different effects on phagocytosis. Three percentage curves were therefore plotted (Fig. 9) showing the observed effect at the end of fifteen, thirty and sixty minutes. These curves are plotted to the same scale as given in Fig. 10.

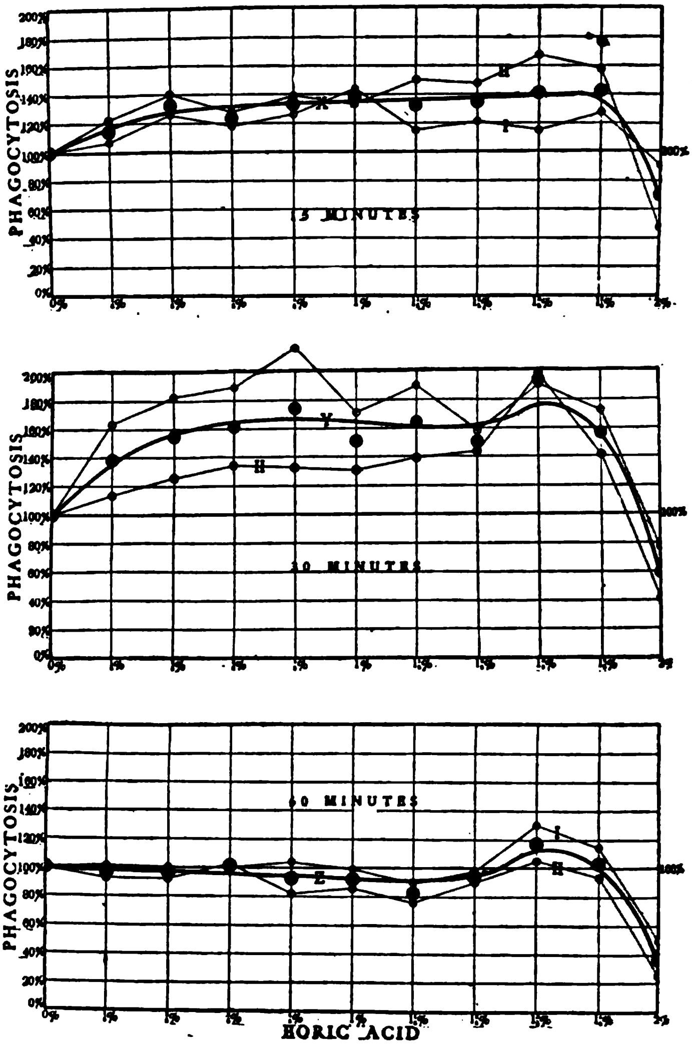


FIG. 9. Influence of boric acid on Wright's index. 15-, 30- and 60-minute data, plotted separately. I = data from first experiment, II = data from second experiment; X, Y and Z = averages.

From Fig. 10 it is seen that boric acid, in small amounts, causes a preliminary stimulation in phagocytosis, followed by a depression. This phenomenon becomes more marked as the percentage of boric acid increases, until a concentration of 1½ per cent. is reached. After this a further increase in boric acid causes a rapid fall in phagocytic power, phagocytosis apparently completely ceasing soon after the concentration reaches 2 per cent.

Quinine Hydrochloride.—The average of the three counts in an experiment with quinine hydrochloride are given in Fig. 11, and

FIG. 10. Influence of boric acid on Wright's index. Curves from Fig. 9, plotted to the same scale.

the corresponding variations in Wright's index in Fig. 12. A duplicate experiment is given in Fig. 13, and the average of the two experiments in Fig. 14.

From this figure it is seen that the addition of quinine hydrochloride gives, from the first, an increase in phagocytosis, the increase reaching a maximum (20 per cent.) as soon as the concentration reaches 1/200 per cent. A further increase in quinine hydrochloride causes a decrease in phagocytosis, phagocytosis being reduced to normal by the time the concentration reaches 1/120 per cent., and apparently completely ceasing soon after the concentration reaches 1/40 per cent.

Whether the observed increase is in the nature of a permanent stimulation, or is only a temporary stimulation to be succeeded by a depression, has not been determined.

Summary.—Judging from counts made at the end of fifteen, thirty and sixty minutes the following influences on phagocytosis have been demonstrated:

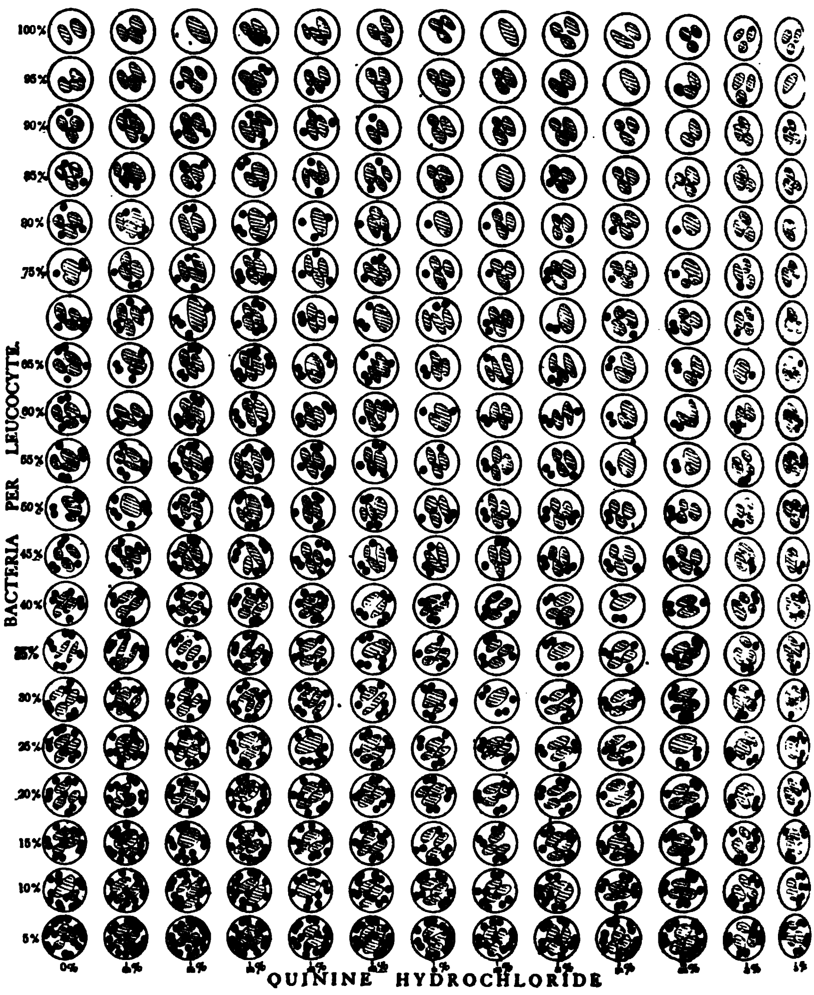


FIG. 11. Influence of quinine hydrochloride on phagocytosis. Human leucocytes; average of 15-, 30- and 60-minute counts; Experiment II.

1. Carbolic acid, added in increasing amounts to experimental tubes, causes from the first a decrease in phagocytic power, phagocytosis falling off one third by the time the concentration reaches

2/9 per cent., seven eighths by the time it reaches 1/3 per cent., and completely ceasing at ½ per cent.

2. Mercuric chloride, in concentrations less than 1/120 per cent.,

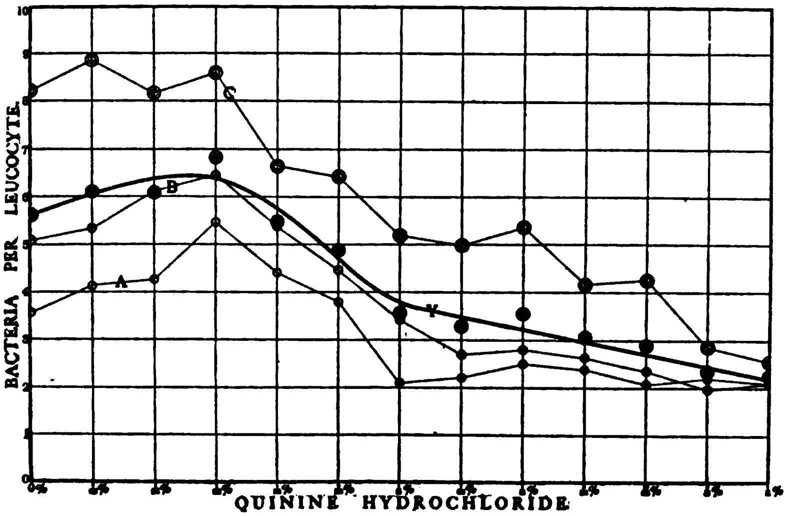


FIG. 12. Influence of quinine hydrochloride on Wright's index. Experiment II. *A, B* and *C* = 15-, 30- and 60-minute counts; *Y* = average.

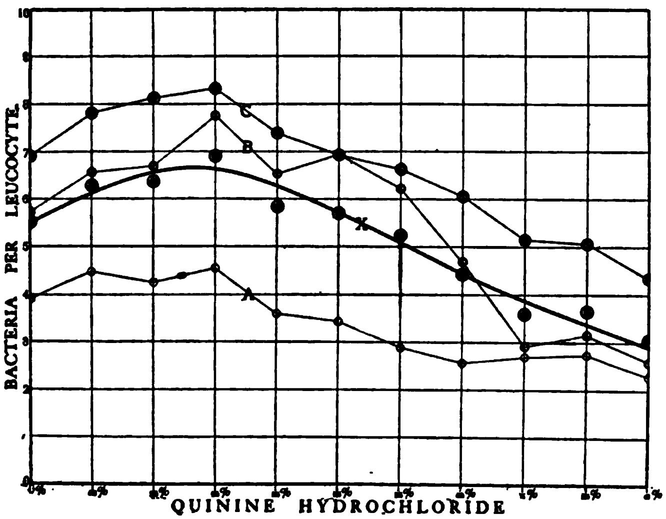


FIG. 13. Influence of quinine hydrochloride on Wright's index. Experiment I. *A, B* and *C* = 15-, 30- and 60-minute counts; *X* = average.

causes a transient stimulation in phagocytosis, followed by a depression. In larger amounts it causes a permanent depression from the first, phagocytosis apparently completely ceasing soon after the concentration reaches 1/60 per cent.

3. Boric acid, in concentrations less than 1½ per cent., causes a transient stimulation in phagocytosis, followed by a depression. As the concentration increases above 1½ per cent. there is a rapid fall in phagocytic power, phagocytosis apparently completely ceasing soon after the concentration reaches 2 per cent.

4. Quinine hydrochloride, added in increasing amounts, causes a

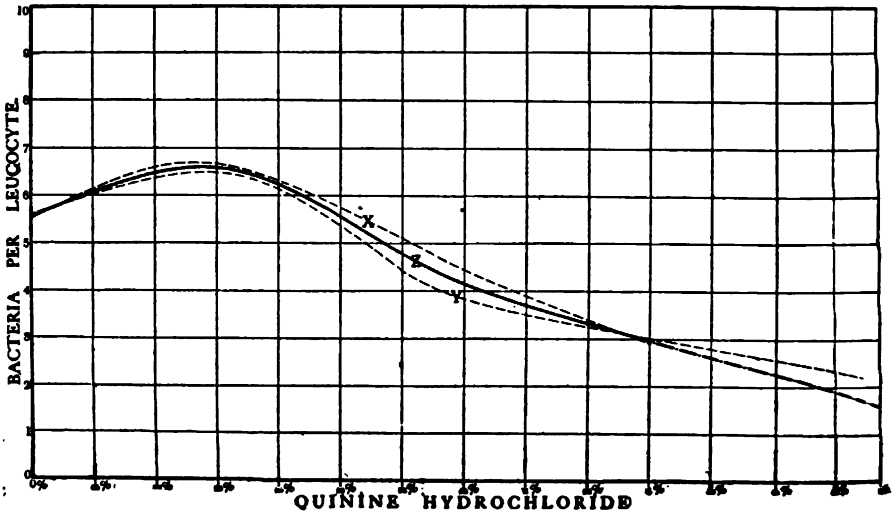


FIG. 14. Influence of quinine hydrochloride on Wright's index. Curves *X* and *Y*, from Figs. 12 and 13, plotted to the same scale; *Z* = average.

stimulation in phagocytosis, phagocytosis reaching a maximum as soon as the concentration reaches 1/200 per cent. A further increase in quinine hydrochloride causes a decrease in this stimulation, phagocytosis being reduced to normal as soon as the concentration reaches 1/120 per cent. In larger amounts quinine hydrochloride causes a depression in phagocytosis, phagocytosis apparently ceasing soon after the concentration reaches 1/40 per cent. Whether the observed stimulation is a permanent stimulation, or a transient stimulation to be succeeded by a depression has not been determined.

A FURTHER CONTRIBUTION TO THE KNOWLEDGE OF THE OPSONINS.[1]

By CHARLES E. SIMON, M.D., Baltimore.

(*From the Laboratory of Dr. Charles E. Simon.*)

In a previous communication I have drawn attention to certain fallacies which attach to Wright's method of estimating the opsonic content of the blood and have suggested that more accurate information may be obtained by diluting the blood in varying proportions and by determining the percentage of phagocyting cells in the resulting preparations. In order to obtain comparable results I proposed the use of maximal numbers of bacteria, as a matter of routine, so that deviations from the normal, in either direction, would be the more striking. The report of the work, in which I was assisted by Drs. R. V. Lamar and Bispham, was submitted to the Rockefeller Institute on the first of March, 1906. Its publication was unfortunately much delayed and it has thus far appeared only in part.[2]

Since that time Wright's work has attracted widespread attention and investigators generally have been following his methods. In the further prosecution of our own work it became necessary, for purposes of comparison, to follow Wright's technique at least in part, and above all to obtain an index which should be directly comparable to Wright's index.

After certain preliminary investigations I then suggested that by comparing the percentage of phagocyting leucocytes in the case of a specimen of blood under investigation with the figure corresponding to a specimen of pooled normal blood serum, terming the latter

[1] Conducted under a grant from the Rockefeller Institute for Medical Research. Received for publication June 20, 1907.

[2] Simon, C. E., and Lamar, V. R. A Method of Estimating the Opsonic Content of the Blood and Other Fluids. *Johns Hopkins Hospital Bull.*, 1906, xvii, 27. Simon, C. E., Lamar, R. V., and Bispham, W. N. A Contribution to the Study of the Opsonins. *Jour. of Exper. Med.*, 1906, viii, 651.

value 1, an index is obtained which is comparable directly to Wright's index. This index I have termed the "percentage index," as contrasted with Wright's "bacillary index." Working with Staphylococcus aureus emulsions prepared with special care and throwing out of the counts cells which had manifestly gotten into clumps (which after all not even with staphylococci can be altogether avoided) I could convince myself that the percentage index and the bacillary index may agree to the second decimal and that the results obtained with either method are directly comparable with each other. This fact having become established it was next ascertained that the percentage index could be used as a direct check upon the bacillary index. It was necessary, however, to work with fairly thin emulsions, viz., such as would furnish values of not much more than 50 per cent. for the normal control blood, in cases at least in which the bacillary index would suggest a material increase of the opsonic content. A normal value of 50 per cent would allow for an increase of the patient's blood to 100 per cent., giving a maximal percentage index of 2. For most purposes this is sufficient; otherwise the blood must be progressively diluted, and the degree of phagocytosis in the diluted specimens compared with correspondingly diluted normal serum. With low values, on the other hand, the emulsions need not be so thin.

A few examples will illustrate the above points:

Example I.—The phagocytic index of the patient was 3.90 and of the normal control 4.88; the opsonic "bacillary" index was accordingly 0.79. The percentage of phagocytosis in the same patient was 60 and of the control 76 and the percentage index hence 0.78, showing the close correspondence of the two indices under favorable conditions.

Example II.—A patient's phagocytic index was 5.11 and that of the control 4.56; the opsonic bacillary index therefore was 1.09. The patient's percentage was 79 and that of the control 72, thus giving a percentage index of 1.09, showing a correspondence of the two indices to the second decimal.

While with especially good emulsions of staphylococci the two indices may thus agree very closely and at times actually coincide, deviations are frequently met with in routine work which show beyond all doubt that Wright's index, when not controlled by the percentage index, may give rise to erroneous conclusions; as a matter of fact results are at times obtained which are absolutely

absurd, and I have no doubt in the least that some of the phantastic curves which have been published are due entirely to errors of technique.

Example III.—The tuberculo-opsonic bacillary index in a case of suppurating bubo, in association with chancroid was 1.93, which according to Wright would have suggested a tubercular process with systemic manifestations. As a matter of fact there was no evidence whatever of any tubercular lesion and the percentage index was normal, viz. 1.18.

Sometimes an increase of both indices shows that there is actually an increased degree of phagocytosis, but the occasionally enormous difference between the two demonstrates that the higher bacillary index has been in part at least owing to the fact that leucocytes had been included in the count which had gotten into clumps and had thus artificially raised the phagocytic and therefore the opsonic index. This error could only have been avoided if in the normal control specimen a corresponding number of leucocytes had gotten into clumps of the same number of bacteria, which of course would have been purely a matter of accident. The suggestion naturally offers itself to exclude from the count cells which have manifestly gotten into clumps. The difficulty, however, would be to decide where to draw the line, which naturally would be more or less arbitrary. Then again it would be necessary to eliminate negative (empty) cells to counterbalance the discarded positive cells, and the question would arise in every case how many negative cells would have to be thrown out from the count for each positive cell, thus giving rise to further difficulties.

Example IV.—The staphylococcus percentage index in a case of sub-phrenic abscess was 1.46 and Wright's index 2.9, whereas dilution to 1:20 showed that there was no evidence of a material increase of the opsonins whatever, viz. 4 per cent., as compared with 8 per cent. on the part of the control.

It might of course be argued that since it is possible *at times* to obtain fairly homogeneous emulsions of organisms, this should be possible at all times, and that accordingly no control of Wright's index is necessary. Practically, however, this is not possible, even with staphylococci, and much less so with other organisms. It seems to me accordingly that a method with which reasonably

experienced laboratory workers cannot obtain uniform and corresponding results must either be abandoned, or it must in some measure be controlled or modified. My experience goes to show that the essential objection to Wright's method and the one which all "opsonic" workers realize is referable to the difficulty, if not the impossibility, of obtaining uniform emulsions. As I have said with staphylococci this is relatively small; with other organisms it is variable, and with the tubercle bacillus almost, if not entirely unsurmountable. Wright himself must realize this, as he has repeatedly changed his technique in this respect, and, it may be added, without announcing such changes, which after all are of fundamental importance. I have worked with extracted and non-extracted tubercle bacilli, with emulsions in 0.1 per cent. and 1.5 per cent. saline, but I have never seen an emulsion yet which was free from clumps.[3]

With other organisms still other difficulties enter into consideration. With the colon and typhoid bacilli, for example, there is usually such a profound degree of destruction with fragmentation and lysis of the organisms and coincident loss of reaction to stains even in normal blood, that a count of the organisms in the leucocytes must of necessity give rise to erroneous results. Even with staphylococci one meets with specimens every now and then, where the staining is very defective and may not be possible at all. I have seen cells filled with colorless "shells" of staphylococci, where a cursory examination would only have shown one or two stained organisms. Counting under such circumstances would have led to the most absurd conclusions, as is shown in the following table. The specimens were sent to me by Dr. North of the College of Physicians and Surgeons, New York, and were of the series which were being investigated by different laboratories for comparative purposes to ascertain the value of Wright's method. As normal control I used the pooled blood of four healthy individuals.

[3] Dr. Cole tells me that he is now working with tubercle bacilli which after cultivation on glycerine agar are killed off by exposure to sunlight, and that with such material he has obtained his best results. I have not yet had an opportunity to examine preparations made with such emulsions.

TABLE I.

Specimen.	Wright's Index.	Percentage Index.
1	.86	.51
2	1.11	.75
3	.81	.44
4	.90	.64
5	.88	.59
6	.88	.55
7	.90	.53
8	1.02	.52
9	1.02	.66
10	1.0	.59
11	.95	.58
12	1.11	.58

The correctness of my conclusions in reference to the accuracy of Wright's method will be borne out on examination by any one who will carefully compare the two indices. But I must insist that *without* the percentage method as a check upon the bacillary method the results obtained with the latter cannot be regarded as trustworthy.

During the past winter I had Dr. Knorr investigate this point in reference to the number of leucocytes that should be counted, and I append the results which were reached. The counts were made with emulsions of varying strength, and with normal blood serum. The results show that the percentage index fluctuates far less normally than the bacillary index. With emulsions containing from 666,000 to 2,000,000 organisms per cubic millimeter the percentage method yields fairly constant results, while with very thin emulsions it also becomes untrustworthy.

TABLE II.

A. *Emulsion of 2,000,000 cocci per c.mm.*

Cells Counted.	Wright's Index.	Percentage Index.
25	1.00	*1.26*
50	.97	1.00
75	.96	1.00
100	*1.69*	1.02
150	1.16	1.12
200	1.23	1.20
300	1.18	1.11
400	*1.27*	1.15

B. *Emulsion of 666,666 cocci per c.mm.*

25	*1.85*	1.18
50	*1.32*	1.16
75	1.03	1.00
100	*1.25*	1.02
150	*1.30*	1.02
200	1.12	.97
300	1.13	1.04
400	1.06	1.00

C. *Emulsion of 222,222 cocci per c.mm.*

25	1.20	*.60*
50	*1.62*	1.14
75	*1.75*	*1.27*
100	*1.75*	*1.26*
150	*2.21*	*1.60*
200	*2.15*	*1.70*
300	*2.00*	*1.62*

D. *Emulsion of 74,074 cocci per c.mm.*

25	*0.66*	1.00
50	*0.50*	*0.62*
75	*0.57*	*0.70*
100	1.14	1.26
150	1.18	1.15
200	0.95	0.96
300	1.05	1.11

As the normal variations in the opsonic index according to Wright may fluctuate between 0.80 and 1.20 it would follow, taking the entire series of examinations, as it stands, that Wright's method gave erroneous results in 50 per cent., while with the percentage method the percentage of error was 30. Throwing out the weaker emulsions we would have 37.5 per cent. of error for Wright's index and only 6.2 per cent. for the percentage index.

The data further show that even a count of one hundred or more cells is no safeguard against error, and that with the percentage index a count of fifty cells is sufficient.

The effect of the thickness of the emulsion upon the bacillary and percentage index is also shown in Table III, which at the same time illustrates how closely under favorable conditions the two will agree; it further emphasizes that the percentage index is a valuable check upon the other.

TABLE III.

No. of Cocci per c.mm.	No. of Cocci per Leucocyte.		Percentage of Phagocyting Cells in		Wright's Index.	Percentage Index.
	(a) Patient.	(b) Pool.	(a) Patient.	(b) Pool.		
3,750.000	2.54	3.15	87	82	.80	.94
1,865.000	1.68	1.90	68	70	.88	.85
937.500	1.26	1.36	58	65	.90	.89
468.750	1.02	1.78	50	45	1.30	1.11
234.75	.73	.71	37	33	1.02	1.05

Working with the two indices side by side during the past winter, I found, as I have said before, that under favorable conditions the two may coincide. If an increase or decrease of Wright's index is associated with an increase or decrease of the percentage index, it may be assumed that such an increase or decrease is not due to errors of technique, and may be interpreted as actually expressing what Wright's index can express. If, on the other hand, the one goes up and the other down, experience has taught me that in such cases the percentage index should be accepted in lieu of the other.

It will be noted that I have just used the expression "what Wright's index *can* express." I do so advisedly, for I am still of the opinion elaborated in a previous paper, that Wright's method does not furnish a proper index of the quantity of opsonic material which is actually present, and I still insist that the dilution method gives results which are more in accordance with the actual facts. I have pointed out that conditions may occur in which the opsonic content of two specimens of concentrated blood serum is apparently the same, but that on progressive dilution the one rapidly loses its opsonifying power, while the other may retain it to a marked extent. This may be observed not only under pathological conditions but at times also in perfect health. I have given examples in one of my previous communications and Amberg[4] has brought out the same point in his studies on the opsonic content of the blood of infants.

It may be objected that in my previous studies emulsions of constant bacterial content were not used, but this, I think, is invalidated by the fact that we always used maximal amounts, and that as a consequence variations from the normal are brought out into

[4] Amberg, *Jour. of Amer. Med. Assoc.*, 1907, xlviii, 304.

bolder relief. After my second winter's work I feel as confident, as I did a year ago, that Wright's method does not furnish a proper index of the opsonic content of the blood, and I am aware of the fact that many of those who have worked upon the same problem have reached the same conclusion. I am not willing, however, to discard the principle of the matter, but would urge other opsonic workers, who stand ready to abandon Wright's opsonic index, to resume their work once more, using my method of dilution. The avowed purpose of taking the opsonic index, aside from diagnostic purposes, is the idea of having a guide to dosage and frequency of inoculation in the treatment of diseases by means of bacterial vaccines. All those who have busied themselves with this subject, and have honestly and without preconceived ideas investigated it, are no doubt ready to admit that the introduction of the bacterial vaccines marks an important epoch in the advance of rational therapeutics. The point at issue at present is the question whether Wright's opsonic index furnishes the desired guide to dosage or not. I, for one, am convinced, as the result of my own work, that in the majority of cases which Wright has suggested as suitable for vaccine treatment, and using the small doses which he has advised, at intervals of one or two weeks, such treatment can be carried out without detriment to the patient, without the use of any index. I have never seen any "negative" phases under such conditions, which could not be perfectly well explained as being due to unavoidable errors of technique. If, however, the attempt at immunization be pushed by materially increasing the frequency of the injections, or by using large doses, I feel that harm may be done and that in such cases even so coarse a guide as the index is better than none. To be sure the physical signs and the patient's general condition may furnish some indication whether the dosage has been too high, but I am a little doubtful in my mind, whether it is desirable to let matters come to such a point, where the physical signs and the general condition of the patient tell us that we have done harm, even though this be but temporary. The only "negative" phases that I have seen have occurred under such conditions, and I feel confident that the result could have been avoided had lower doses been used first and controlled by the index.

The following case will serve to illustrate this point.

A little boy of eight, the subject of recurrent epulis, was injected with 575 millions of Staphylococcus aureus on Nov. 20th; his index on the 13th had been .62 (bacillary) and .80 (percentage); on the 15th it was 1.4 (bacillary) and 1.0 (percentage), and on the day of the injection 1.6 (bacillary) and 1.1 (percentage). During the night of the 20th to the 21st his temperature rose to 103°, followed by a profuse sweat, and on the 27th the index had dropped to .30 (bacillary) or .45 (percentage). By December 4th the two indices had risen again to .80 and .90 respectively, and on the 11th they were 1.0 and .80. On that day an injection of 600 millions was given, which was followed by a drop to .10 and .22 respectively (see Curve I).

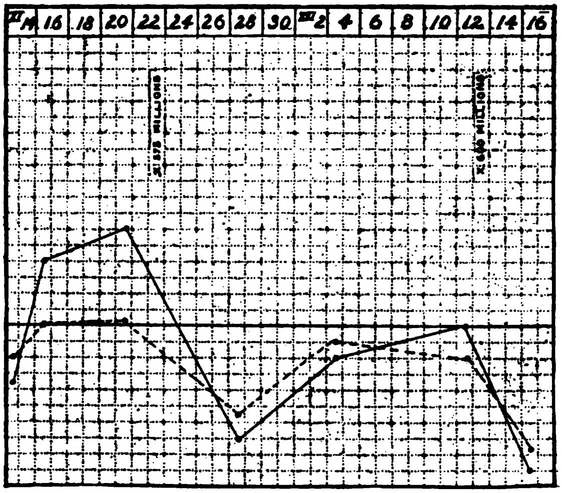


FIG. 1. Solid line indicates the bacillary index; dotted line indicates the percentage index.

Drops in the opsonic index such as these, following upon the injection of bacterial vaccines, occur very rarely in my experience, if small doses are used. They do occur, however, and I personally have the conviction, that it cannot be a matter of indifference to the patient, if his phagocytic power is practically brought to nil, and especially so in those infections in which the process of phagocytosis is one of the main stays of resistance.

During the past winter I have had occasion repeatedly to use bacterial vaccines in *acute* staphylococcus infections, and I may add, at times with very good results. The clinical course in these cases made it clear that injections given a week apart would have been without avail, and an attempt was therefore made to stimulate

the mechanism of immunization by pushing the vaccine. The question naturally was what guide to dosage should be used, and, in lieu of a better, the opsonic index was followed. These cases will be reported in detail at another occasion, but it may not be out of place to cite at least one.

The patient was a young women of about twenty-five, who a week after childbirth was seized with a chill with a temperature elevation to 104.1°; the next day she had another chill with a temperature of 104°. From this day her temperature varied between 101° and 105°. I saw her for the first time on the evening of Jan. 21st, eight days after the onset of the fever. At that time the temperature was 104.8°; she was quite somnolent and made the impression of a very sick woman. The physician in attendance had isolated Staphylococcus aureus, and she was accordingly injected with aureus vaccine. One thousand millions were given on the evening of the 21st and another dose the following morning. Her temperature promptly came to normal and remained so until the 26th. On that day and the following there was an evening rise to 102.5°. She received 750 million cocci on the evening of the 27th, and the same dose on the morning of the 28th. Her temperature promptly again came to normal and remained so. Her index (percentage) at the time of the first injection was .95; the next morning 1.0 (no negative phase) and on the 23d, viz. 36 hours after the first and 24 hours after the second injection, 1.90. The further course is seen in the curve (Curve II).

Fig. 2. Solid line indicates highest and lowest temperatures of the day: dotted line is the opsonic curve.

I have been surprised to see to what extent the injection of the staphylococcus vaccines can be carried in some cases, and manifestly not to the detriment of the patient. In one of my acute

cases 1,000,000,000,000 were injected within a week, and I may add without the appearance of a negative phase, and with an excellent effect upon the patient's condition (staphylococcus septicæmia with multiple abscesses). Nevertheless I hardly feel willing to carry the injections to such a point without some guide, better than the physical signs and the patient's general condition, and I feel convinced that the opsonic index in such cases is of service. But I repeat once more *with small doses,* such as Wright recommends, *administered at intervals of a week or two in chronic, well localized infections the opsonic index is of little, if any real value, either as a guide to dosage or to the time and frequency of inoculation.*

THE SPECIFICITY OF THE OPSONINS.

As regards the specificity of the opsonins my earlier investigations led to the conclusion that under normal conditions at least a specificity of the opsonins does not exist,[5] and I suggested then that it is quite conceivable that as a result of infection, viz., immunization substances may be produced either *de novo,* or increased in amount, if preëxistent, which may so influence a given organism that under the subsequent action of a common non-specific opsonin, it is taken up in larger or smaller numbers, as the case may be.

During the past winter I have repeated many of the experiments of the previous year, to eliminate possible errors of technique, but have been forced to the same conclusion, viz., that *the opsonins of normal blood serum are non-specific.*

This conclusion is based upon absorption experiments which were analogous to those previously reported. Since some of the organisms, however, and notably when relatively avirulent, are taken up by the leucocytes already in physiological salt solution (Bacillus subtilis, Bacillus pyocyaneus, Bacillus coli), and since some of Bulloch and Western's[6] contrary results may possibly be explained upon this basis, I chose only such organisms which had recently been isolated from infected individuals. Working with these I could find no evidence whatever that absorption of the opsonins for one organism leaves opsonins for other organisms behind. To be

[5] *Jour. of Exper. Med.* 1906, viii, 651.
[6] Bulloch, W. and Western, G. J., *Proc. Royal Soc.,* 1906, lxxvii, 531.

sure I never carried the absorption to a point where opsonic action ceased altogether, but in all cases there was a marked drop for both.

Example I.—About 0.5 c.c. of normal blood serum was inoculated with one-half of a twenty-four hour staphylococcus agar culture, and incubated for twenty minutes at 37° C. The percentage of phagocytosis before inoculation was 100 for the staphylococcus and 90 for the colon bacillus. After incubation the serum was centrifugalized at high speed for one hour, the supernatant fluid transferred to a new tube and centrifugalized for an additional half hour. The percentage of phagocytosis, for the same emulsions, was then 60 for the staphylococcus and 50 for the colon bacillus.

Example II.—In another experiment the values before absorption were 64 for staphylococcus and 96 for colon, and 44 and 40 respectively after absorption with the staphylococcus.

Example III.—In this instance the staphylococcus value before inoculation with staphylococci was 96 and the colon value 64, while at the end of the experiment they were 16 and 20 respectively.

While my results thus do not agree with those of Bulloch and Western it is possible, as I have pointed out, that the phagocytosis which they noted for the non-absorbing organism after absorption with the other, may have been due to non-virulence of the bacteria used and consequent spontaneous phagocytosis. In one class of his experiments Bulloch uses Bacillus pyocyaneus after absorption of the serum with staphylococci, and finds that the serum has lost its opsonic power for the staphylococcus, while that for the pyocyaneus is preserved. This is not at all surprising since laboratory cultures of the pyocyaneus are taken up very readily in physiological salt solution.

I have not used the tubercle bacillus in my experiments, as living virulent organisms were not available.

Bulloch's observations that inoculation of a human being with tuberculin or with staphylococcus vaccine causes a quantitative increase in the tuberculo- and staphylococcus opsonins respectively, while the staphylococcus and tubercle opsonins in turn remain unaltered, are not well adapted to either prove or disprove the point at issue. As a result of the injection of bacterial vaccines the entire immunization mechanism of the body is thrown into activity and immune opsonins possibly formed at the same time. So long as the identity of normal and "immune" opsonins, however, has not been proven it seems to me that conclusions based upon the for-

mation of the latter are not applicable in the discussion of the specificity of the opsonins of normal serum. I should like to remark nevertheless that the injection of either tubercle or staphylococcus vaccine in the small doses recommended by Wright, does not necessarily cause an increase in the opsonic index in reference either to the one organism or the other.

Example I.—Mr. C. medical student. Staphylococcus index on Jan. 29th was .90. He was injected with 750 millions of staphylococci on Jan. 30th and on Dec. 2d the index was 1.15.

Example II.—Mr. R. Staphylococcus index on Jan. 10th .95; injection of 700 million staphylococci; index on the 12th 1.04.

Example III.—Miss R. Tubercle index on Jan. 29th .83; injection of 1/1000 mgrm. of tuberculin on the 30th; index on Feb. 2d .90.

These observations thus do not tend to bear out the correctness of Bulloch's view that the opsonins of normal serum are specific and my experiments on the blood of different animals, not only of different species but of the different classes of vertebrates in general, in all of which I could demonstrate the presence of opsonins for various organisms, similarly point to the same conclusion. Particularly striking is the fact that in the lower classes of vertebrates I could demonstrate the presence of opsonins for organisms with which infection does not occur in these animals. I recall the marked opsonifying power of the blood of the terrapin, of the frog and of fowls for Staphylococcus citreus, as an example.

Passing from the consideration of the specificity of the opsonins in normal blood to that of the blood in the infected individual there is evidence to show that here actual specificity may exist, but I must add, does not necessarily exist. While under normal conditions the opsonic content of the blood varies within relatively narrow limits, the variations in infections are much wider. These limits, so far as the "index" goes, Wright and his coworkers have placed at 0.8 and 1.2. My inclination would be to extend them somewhat more, for I have found indices as low as 0.70 and as high as 1.25 in individuals who showed no evidence whatever of any infection. At the same time I must admit that this is exceptional. This question is an important one in deciding from how many individuals blood should be taken to make up a normal control serum. I had Dr. Knorr investigate this point with the result

While then the opsonic index in normal individuals (barring women during the menstrual period, and the exceptional individuals of whom I have spoken) varies within comparatively narrow limits, most remarkable deviations from the normal figure may be met with in disease. In their earlier work Wright and Douglas[7] reported a series of staphylococcus infections (furunculosis, sycosis, acne, etc.) in all of which the opsonic index was abnormally low, ranging from 0.1 to 0.87. They add that they have not come across any instance of the association of a normal phagocytic power with a staphylococcus infection. Analogous results were obtained in cases of tuberculosis. In one series of seventeen cases the values ranged between 0.4 and 0.85, including cases of tubercular peritonitis, laryngeal phthisis, psoas abscess, lupus, pulmonary phthisis, etc. In a series of twenty-five cases of pulmonary tuberculosis other than the acute form, Lawson and Stewart[8] also found low figures, on the whole, although in some the values were but little, if at all, below the normal average, viz., 0.5 to 1.0.

Subsequently Wright pointed out that low values are the rule in strictly localized infections, while in systemic infections abnormally high values may be observed.

As the index in the various infections shows deviations from the normal which usually only affect the offending organism, and as the injection of bacterial vaccines in such cases has a direct effect upon the corresponding bacterial index, Wright and his colaborers conclude that these facts indicate that the opsonins in the infected organism are specific. As they do not admit that a material difference exists between the opsonins of normal serum and the serum of infected individuals they further conclude that the normal opsonins also are specific. I have pointed out that there is no satisfactory evidence to support this view.

In studying the question of the specificity of the opsonins in the infected organism, which we shall refer to for the present, as a matter of convenience, as " immune opsonins," a series of absorption experiments were undertaken which were perfectly analogous to those described before, but in which the serum of infected indi-

[7] Wright and Douglas, *Proc. Royal Soc.*, 1904–5, lxxiv, 147.

[8] Lawson and Stewart, *Lancet*, 1905, ii, 1679.

viduals was exhausted, viz., of persons and animals which had been treated with bacterial vaccines. My experiments thus far have reference largely to staphylococcus and typhoid infections, but in neither could I find any evidence that absorption with one organism removed a homologous opsonin and left a heterologous opsonin behind. Working with dried and pulverized tubercle bacilli, on the one hand, and staphylococci, on the other, I have obtained results which corresponded in a way to those of Bulloch, viz., after absorption with staphylococci I occasionally found a slightly higher value for the tubercle bacillus than for the staphylococcus. If the experiment was reversed, however, the tubercle value was still higher. This leads me to think that the causes of the higher retention lay in the powdered bacilli and not in the serum, and that a different result might have been reached, if live virulent tubercle bacilli had been used instead. But, as I have said, such were not available at the time. As far as they go, however, my absorption experiments do not bear out the existence of specific "immune" opsonins.

A further argument which has been advanced as supporting the idea of the existence of specific opsonins, has reference to the greater thermolability of the normal opsonins as contrasted with the thermolability of the opsonins of "immune" sera. Wright, to be sure, does not admit that a material difference exists between the two, for he says:[9] "Manifestly the plain teaching of our experiments is, that the opsonin which is found in the heated immune serum of a patient who has responded to tubercular infection (I assume that his remarks would apply to any other infection in which phagocytosis plays a *rôle*), or as the case may be to the inoculation of a tubercle vaccine, does not differ, with respect to its resistance to heat and sunlight, from the opsonin which is found in the unheated normal serum. A precisely similar conclusion with respect to the identity of the opsonins found respectively in unheated normal and heated immune sera. . . ."

Other observers do not agree with Wright in his assumption that the opsonins of normal and immune sera are identical, notwithstanding his attempted explanation to this end. He himself, how-

[9] *Proc. Royal Soc.*, 1906, lxxvii, 224.

ever, manifestly attaches special significance to the fact that the opsonins of immune sera may be thermostable, or, as it would possibly be better to say, that they contain a thermostable component. The fact that he suggests that the thermostability of the opsonin toward a single organism may be used for diagnostic purposes shows that in his mind this thermostability is intimately associated with the question of specificity. In two tables he gives examples illustrating the thermostability of the opsonins toward the tubercle bacillus and introduces them with the remark that they represent a typical selection from a very extensive body of observations which furnishes the basis for the preceding statement as follows: "When a serum is found to retain in any considerable measure, after it has been heated to 60° for ten minutes, its power of inciting phagocytosis, we may conclude that 'incitor elements' have been elaborated in the organism, either in response to auto-inoculations occurring spontaneously in the course of tubercular infection, or as the case may be, under the artificial stimulus supplied by the inoculation of tubercle vaccine." This is important, as Wright himself thus restricts the drawing of any diagnostic inferences from absence of thermostability. It is remarkable, however, on the one hand, that many cases do occur in whom there is every evidence of systemic infection, without thermostability of the opsonins, and that thermostable opsonins, on the other hand, may be found in persons in whom there is no evidence of infection.

TABLE VI.

Showing a series of cases in which active phagocytosis was not obtained after heating the sera for 10 minutes to 60° C.

Number.	Nature of Infection.	Unheated Bacillary Index.	Serum Percentage Index.	Heated Bacil. Index.	Serum Percent. Index.
Miss R.	Vesical tuberculosis.	.57	.66	0	0
Mr. G.	Tuberculosis of cæcum.	1.10	.91	.08	.03
Mr. G.	Tuberculosis of cæcum.	.95	1.08	.17	.25
Mr. B.	Renal tuberculosis.	.75	.80	0	0
Miss M.	Tubercular conjunctivitis.	—	.81	—	.09
R.	Staphylococcus suppuration.	.53	.94	.03	.20
Miss Sch.	Staphylococcus septicæmia.	.08	.27	.03	.11
Miss Sch.	Staphylococcus septicæmia.	2.36	1.53	.08	.26
G.	Abscess of finger (staph.)	1.63	1.26	.06	.06
Z.	Drained pelvic abscess (staph.)	1.36	1.20	.06	.06
M. D.	Tubercular hipjoint.	1.2	.9	0	0
X.	Tubercular elbow joint.	—	.85	—	0

Of cases in which marked thermostability was observed without any evidence of infection of any kind I may mention two. In the first there was from time to time hæmaturia of slight grade without any apparent cause. The patient was examined in great detail by Dr. H. A. Kelly, with negative result; tubercle bacilli could not be demonstrated in the urine; an injected guinea-pig remained well and the tuberculin test was negative. The tuberculo-opsonic index on three successive days varied between .52 and 1.20; corresponding to the latter value the index with the heated serum was .39.

In the second case there was likewise no evidence of tuberculosis with the usual methods of examination; the tuberculo-opsonic index, however, varied between .36 and 1.6 with the unheated serum and between .26 and .45 with the heated.

In studying these negative cases and contrasting them with my positive cases and with those of Wright, I cannot help but feel that in some of the positive cases the reaction in question is specific, but that in the negative cases we are dealing with normal non-specific opsonins. The difficulty may lie in our present inability to differentiate between infection *per se* without immunity production and infection associated with immunity production. This is even more so the case when we study the opsonic index toward a given microorganism from the standpoint of differential diagnosis, aside from the question of thermostability. Wright's conclusions that deviations from the normal values may be interpreted as evidence of infection with the homologous organisms suggest very strongly that a specificity exists, but it is difficult to understand, on the other hand, why so many cases of infection do not show any material deviation from the normal and cases, moreover, in whom there could be no doubt that systemic reaction was going on. Table VII shows a series of such cases.

In one case of acute staphylococcus septicæmia which ended fatally on December 15 and in which daily observations had been made since December 8, an abnormal index was only noted on two occasions, viz., .40 on December 8 and 2.0 on the twelfth; on the remaining days the values were entirely normal.

While my observations bear out the correctness of Wright's conclusions in a general way, that deviations from the normal for a

TABLE VII.

Showing a series of cases of both localized and systemic infections in which no material deviation from the normal-homologous index was obtained.

Name.	Disease.	Percentage Index.
Miss W.	Acne	1.0
Mr. B.	Renal tuberculosis	1.25
Mr. G.	Tuberculosis of the cæcum	1.25
L. B.	Glandular tuberculosis undergoing treatment with with tubercle vaccine	.90
S.	Tubercular hipjoint	1.10
J.	Tubercular hipjoint	.87
C.	Suppuration following operation	1.00
Dr. S.	Furunculosis	.75
Sp.	Suppuration following burns	.93
R.	Suppuration following operation	.94
W.	Staphylococcus abscess of neck	.88
J.	Suppurating bubo	.81
W.	Tubercular adenitis	1.18
F.	Staphylococcus septicæmia (multiple abscesses)	.90
L.	Staphylococcus septicæmia	.95

given organism may be regarded as important evidence from the standpoint of differential diagnosis, normal values can manifestly not be interpreted as excluding the possibility of infection with the organism under consideration.

But, as I have just said, my conclusion in reference to deviations from the normal coincide with those of Wright, only in a general way, for I have seen marked deviations both upward and downward for various organisms in cases in which there was not a corresponding infection; and the fact, moreover, that in some of these cases, at any rate, the deviations had reference not to a single species of organism seems to me to be further evidence to show that the opsonins in question were not specific.

One case in point (Mason) was one of syphilis of the liver with ascites; there was no evidence whatever of tuberculosis, while the tuberculo-opsonic index was .27.

In a second case of hepatic syphilis with ascites both the tuberculo-opsonic index and the staphylococcus index were increased, 1.4 in the case of the former and 1.3 in the latter (the bacillary indices were 2.6 and 2.3 respectively).

In a case of sarcoma of the breast and the axillary glands the tubercle index was .40 and the staphylococcus index .73 (the bacillary indices .39 and .37).

A typhoid patient in the second week of the disease gave a staphylococcus index of 1.46 (1.85 bacillary), in the absence of any apparent staphylococcus infection.

I further append some staphylococcus citreus values, obtained with my method of dilution, in cases in which a citreus infection could hardly be assumed to have existed.

TABLE VIII.

Disease.	1:20 Dilution.	1:30 Dilution.	1:40 Dilution.
Normal (average).	37.2	19.4	9.2
Appendicitis.[10]	96	88	—
Myelogenous leukæmia.[11]	—	6	0
Syphilitic ulcer of cæcum.	18	0	0
Carcinoma (generalized).	0	3	0
Appendicitis.	96	92	80
Retroperitoneal sarcoma.	—	68	64
Syphilitic cirrhosis.	100	96	—
Pneumonia.	100	92	88
Pneumonia.	100	88	84
General carcinomatosis.	100	92	64
Appendicitis.	100	100	100
Pneumonia.	100	88	60
Appendicitis.	20	—	0
Beri-beri.	4	0	0
Ruptured extra-uterine pregnancy..	12	4	0
Melanotic sarcoma.	8	—	0
Pernicious anæmia.	92	42	28
Spleno-medullary pseudo-leukæmia.	88	60	20

With these various facts before us, I believe, the conclusion is justifiable that *granting the opsonins to be definite entities their specificity, even in cases of infection has not yet been established, although certain data seem to point in that direction.*

OPSONIC IMMUNITY.

From the writings of Wright the majority of his readers no doubt have gained the impression that as a consequence of successive inoculations of bacterial vaccines an opsonic immunity gradually becomes established, which would be characterized by a persistingly high index, a continuous "high tide phase" to use Wright's own expression. Unfortunately there are no exact data published by Wright to throw light upon this question. My own investiga-

[10] I have included appendicitis cases in this table, since staphylococci are only very rarely found under these conditions and there is little reason to assume that they were staphylococcus infections.

[11] This low value was obtained during a period of great improvement in the patient's condition with practically normal blood picture.

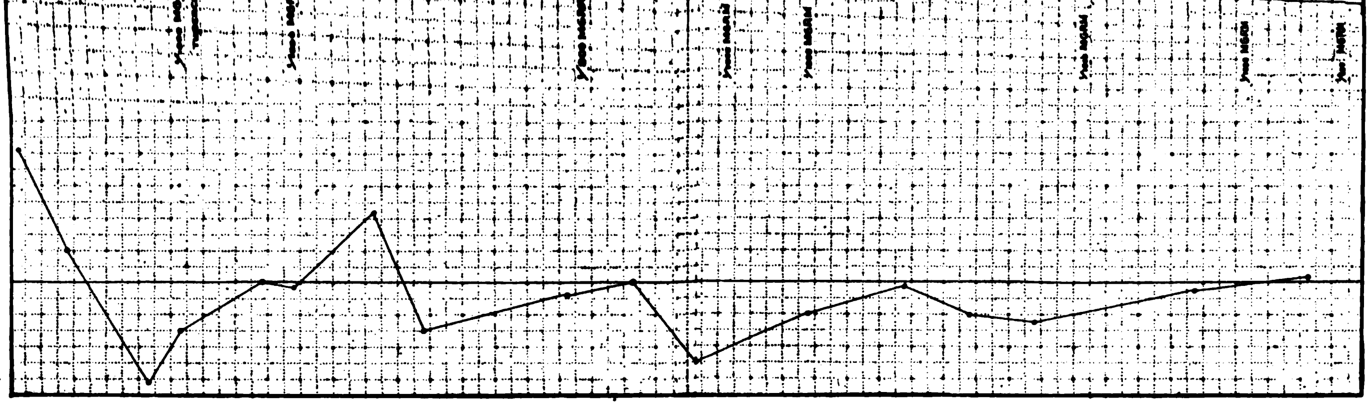


FIG. 3. Miss A. Vesical tuberculosis; recovery under treatment.

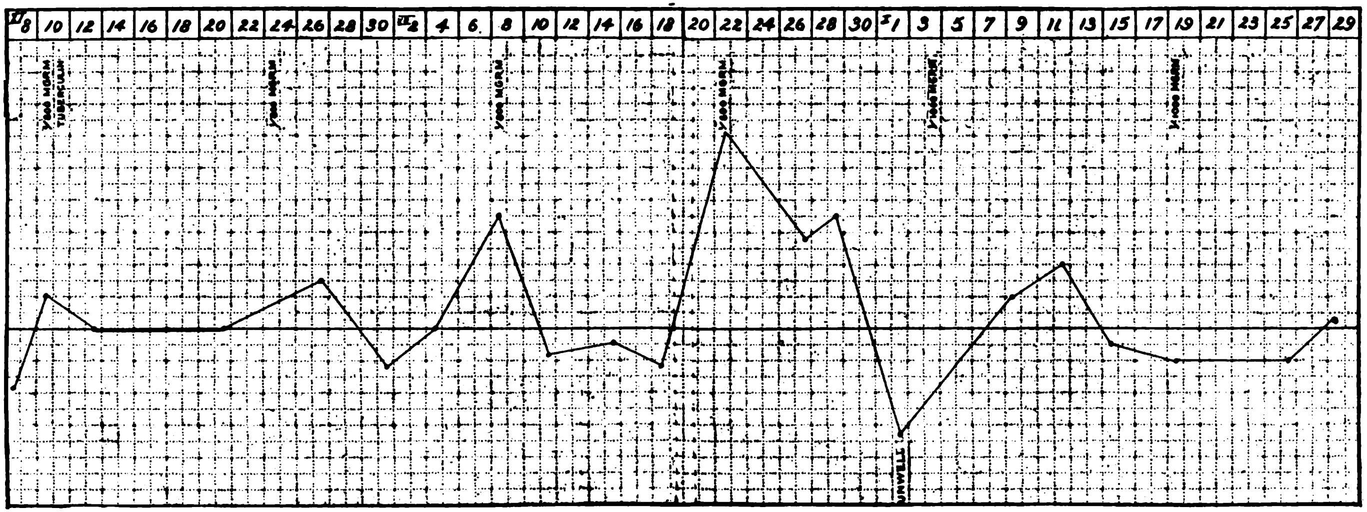


FIG. 4. Mrs. N. Vesical tuberculosis; under treatment nearly a year with great improvement.

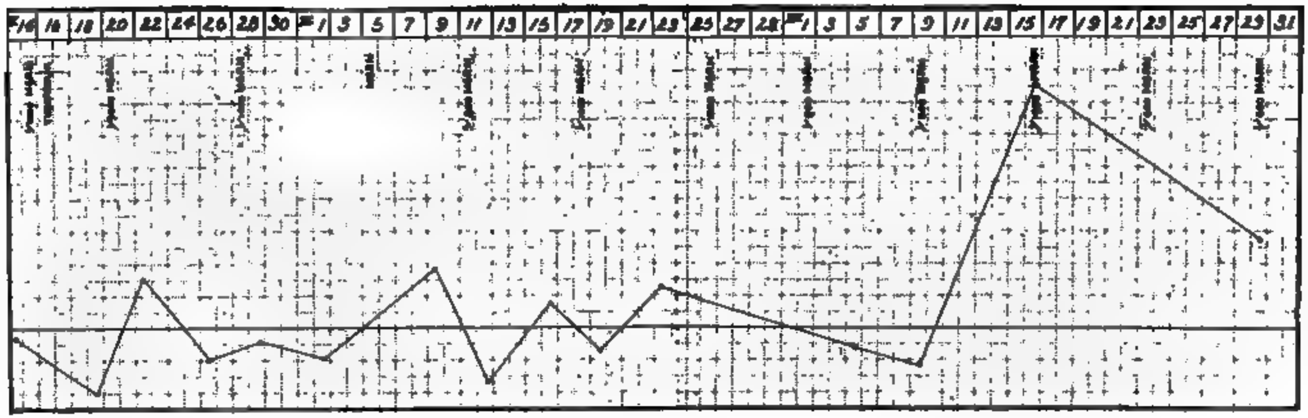
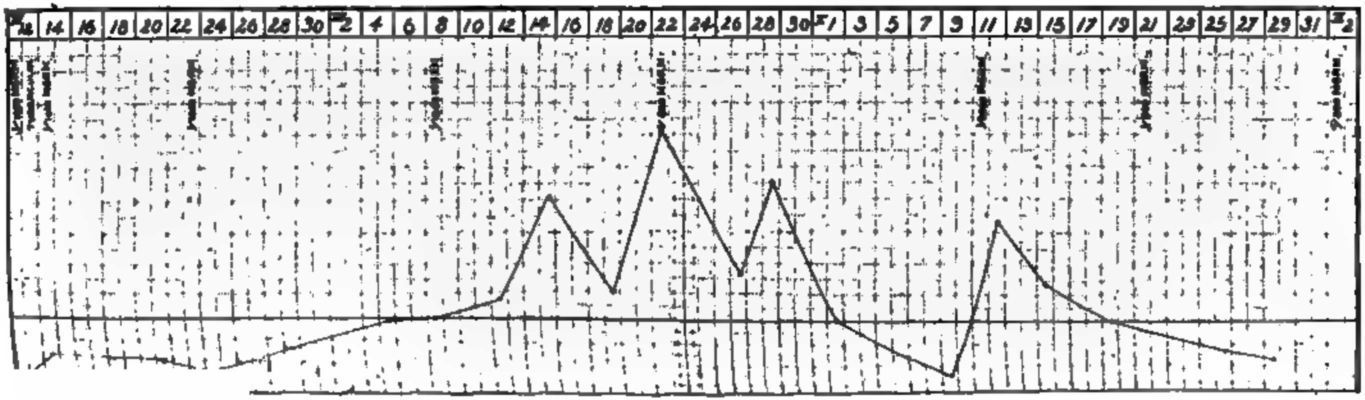


FIG. 5. Mr. B. Tubercular horse-shoe kidney and calculous pyelitis; gain in weight under treatment, but tubercle bacilli continue in the urine.



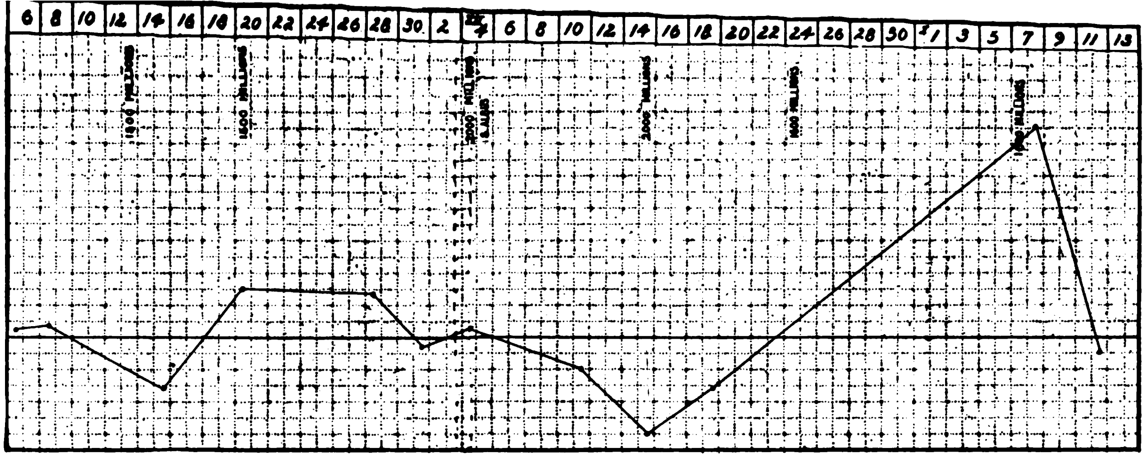


FIG. 7. Mr. A. Acne; recovery.

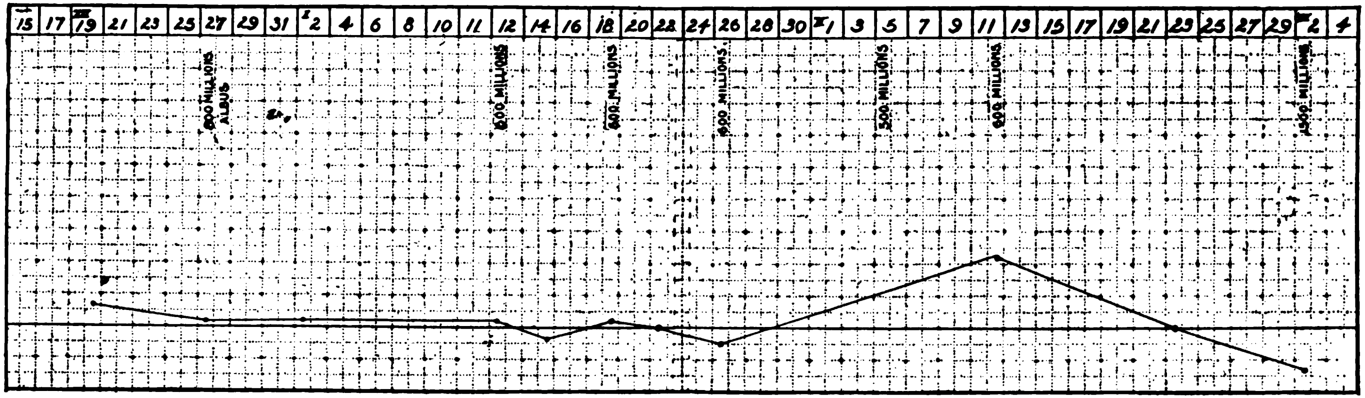


FIG. 8. Miss F. Acne; recovery.

tions in this direction have reference almost exclusively to tubercular and staphylococcus cases. I have already pointed out that many cases, of this kind, occur in which the index at the very outset of immunization is not materially lowered and that no very marked fluctuations may occur. In such cases successive inoculations of the corresponding vaccines brought about no material change in the height of the index, as compared with the normal. With small doses no negative phases were observed which could not readily be explained as being due to unavoidable errors of technique. Within a day or two there was usually (but not always) a rise in the index which then dropped again to near the normal line (Curve III, IV and V). A distinct and persistent rise was only observed in cases in which the nutrition of the patient was markedly below the normal at the start. As this improved (during the process of immunization) the index gradually rose to normal, without any marked fluctuations. In one instance (Curve VI) remarkable variations then occurred (the patient steadily improving), but it will be noted without a persistingly upward tendency.

In no instance was a continued high tide observed and after several months of active immunization the index was not found higher than normal (Curve VII and VIII). The patient from whom Curve IV was taken had been under vaccine treatment for nearly a year.

Animal experiments led to the same result. We may accordingly conclude that in the case of the tubercle bacillus and the staphylococcus a study of the opsonic content of the blood by Wright's method shows no evidence of the development of an opsonic immunity, which likewise argues against the specificity of the opsonins. The available literature having reference to this point is still too meager to furnish any data which could be interpreted as evidence either for or against this conclusion, but I note that Bulloch states that "in most cases the high tide is in the course of a few days succeeded by a fall again. . . . In spite of this the improvement may continue, a fact which leads me to assume that there are other factors at work which at the present time cannot be measured." This coincides with my own views and I would emphasize that no matter what the future of the opsonic "index"

may be there can be no doubt whatsoever that remarkably beneficial results can be reached by means of bacterial vaccines and that Wright deserves full credit for having generalized this principle of immunization in human therapeutics.

OPSONINS AND AGGRESSINS.

In order to ascertain whether the lowered opsonic content which may be observed in the various bacterial infections is referable to an insufficient production of opsonic material or to the simultaneous presence of inhibitory substances (aggressins, endotoxins), a series of experiments was undertaken in which the blood of infected individuals was added to normal blood and the phagocytic power of the mixed blood compared with the normal. My results are not yet numerous enough to warrant any definite conclusions, but they show that in some instances in which the patient's index was low, a distinct inhibitory effect occurs. That this is not the expression of dilution merely, with a serum of lowered bacterial content, is clear from the fact that the substitution of saline solution for the patient's blood does not cause a corresponding effect.

Example I.—The patient was a young girl with generalized septicæmia following an appendectomy. Her opsonic content on Nov. 25, measured by my percentage method, was 18; the normal control value was 72, and her percentage index accordingly .25. A mixture of equal volumes of the control serum and saline solution gave 32 per cent., corresponding to an index of .44, while a mixture of the patient's and the control serum gave only 16 (index .22). There was thus a drop of 50 per cent., which, I think, cannot be otherwise interpreted than as being due to the action of inhibitory substances in the patient's serum.

Such results, however, were exceptional and not constant even in one and the same individual. At times, indeed, the mixed sera gave higher values than the control, conveying the impression as though the normal serum had liberated a bound quotum of the patient's opsonins. If this idea of bound opsonins in the infected individual could be shown to hold good, it would explain the curious fact that notwithstanding the existence of such infections in which the process of phagocytosis is the only method of defense of which we have evidence, normal values are so frequently observed although active symptoms exist, from which we would expect to find the opsonins either high or low, but certainly not normal.

Example II.—On the sixteenth of February the percentage value of the patient referred to in Example I was 20, while the control was 72; her index hence was .27. The mixture of control and saline gave 40, *i. e.*, an index of .55, while that of the patient's and the control serum was 80, corresponding to an index of 1.11 and representing an increase of 100 per cent.

Example III.—In a woman following hysterorrhaphy a mixed staphylococcus and streptococcus septicæmia developed. Her percentage value for the staphylococcus was 76, while the control was exactly the same and her index hence 1.00. The mixture of the control and saline gave 60 (index .78), while that of the patient's serum and the control was 92 and the index hence 1.21, representing an increase of about 50 per cent.

Working with exudates I have repeatedly noted that their addition to normal serum causes a more marked drop in the phagocytic index than the corresponding blood serum. This is manifestly not due to a lower content in opsonic material *per se,* as transudates and cystic fluids likewise furnish lower values; but their admixture (when fresh) to normal serum does not bring about a corresponding decrease.

Example IV.—The percentage phagocytic value of a specimen of fresh hydrocele fluid was 12, while that of the normal blood serum was 72; the index thus was .16. The mixture of control serum and saline gave 52 (index .72) and that of the serum and hydrocele fluid 48, corresponding to an index of .66, thus showing no material effect.

Example V.—A specimen of fluid was obtained from an intraligamentary cyst; its phagocytic value was 16 per cent. and that of the control serum 76; the index hence .21. Equal parts of serum and saline gave 42 per cent. (index .55) and of saline and cystic fluid 48 per cent. (index .63).

Example VI.—Fluid from an ovarian cyst; phagocytic value 4 per cent. (index .10); equal parts of control serum and saline gave 32 per cent (index .80) and of saline and cystic fluid 28 per cent. (index .90).

There is thus no evidence of any inhibitory action whatever. But with exudates, as I have said, there is often a marked effect upon the phagocytic power which I hardly think can be interpreted otherwise than as being due to definite inhibitory substances.

Example VII.—Mrs. C., the patient referred to before (Example III). The staphylococcus value of the pleural exudate in this case was 48 (index .63), while with the streptococcus no phagocytosis whatever was observed. It is interesting to note that the exudate contained streptococci in large numbers, which were cultivated from the exudate and from the patient's blood, while the staphylococcus infection was apparently localized. The mixture of normal serum and saline for the staphylococcus gave 76 and for serum and exudate 80 (index 1.05), while with the streptococcus the values were 42 and 12 respectively, giving an index of .23, thus showing a decrease of over 70 per cent. (!), which, as I have said before, must be attributed to the action of inhibitory substances.

The following example shows a similar effect.

Example VIII.—The patient was an old gentleman, aet. about 70, with empyema. The phagocytic power of his blood serum was 88 per cent., corresponding to a control of 72, thus giving an index of 1.11; the value of the exudate was 12 and the index hence .16. Equal parts of normal serum and saline gave 60 per cent. (index .88) and of normal serum and exudate 12 per cent. (index .16) showing a drop of about 80 per cent.!

The patient died a few days later and the exudate which was drawn off after death again examined. Its direct phagocytic value was 4 (index .10); normal serum and saline gave 32 (index .80) and saline and exudate 8 (index .20), thus showing a decrease of 75 per cent.!

Example IX.—(Same patient as Example I.) Within forty-eight hours preceding death ascites developed and in the exudate chain cocci, diplococci and bacilli were found in immense numbers. Some of the material was freed from bacteria by prolonged centrifugation and then showed a staphyloccus value of 4 per cent. (index .16); normal serum and saline gave 16 per cent. and with serum and exudate no phagocytosis whatever occurred—a loss of 100 per cent.!!

Of the character of the inhibitory substances in the blood and in the exudates of infected individuals nothing is known. I have some evidence, which suggests that they are comparatively unstable, but am not prepared to discuss this phase of the subject at the present time. Whether or not they are of bacterial origin and of the nature of aggressins (viz., endotoxins), or whether they are products of autolysis referable to the cells of the infected individual future research will have to show, but their demonstration in the blood and exudates renders it possible, if not probable, that the low opsonic values which are so frequently seen in the various infections may be due in part at least to the simultaneous presence of antagonistic substances.

CONCLUSIONS.

1. The determination of Wright's index of the opsonic content of the blood and other fluids of the body is open to serious and in part unavoidable errors and should be abandoned in its present form.

2. Conclusions based upon the determination of the opsonic content of the blood, according to Wright's method, are accordingly not uniformly reliable.

3. The percentage index is a valuable check on Wright's bacillary index, but likewise does not furnish an adequate idea of the opsonic content of the blood, unless carried out with progressive dilutions to the point of opsonic extinction.

4. The opsonins of normal blood serum are not specific.

5. The specificity of the opsonins in "immune" sera has not been satisfactorily established, but appears probable.

6. An opsonic immunity, in the sense of a continued high opsonic content of the blood does not exist.

7. In the blood and exudates of infected individuals substances may be present which exercise an inhibitory effect upon phagocytosis.

OPSONINS OF INFLAMMATORY EXUDATES.[1]

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There is much uncertainty concerning the relationship of so-called opsonins to other bodies concerned in the production of immunity and available data is insufficient to indicate with what accuracy the opsonic power of the serum measures resistance to bacteria either in normal or in immunized individuals. Wright has found the opsonic power of the serum with certain bacterial infections much below the normal and believes that a low opsonic index indicates susceptibility to the particular microörganism which has been employed in testing the opsonic activity of the blood serum. The low opsonic index observable with certain pyogenic and tuberculous infections is believed by Wright to antedate infection, being indeed its cause. There can be no doubt that the opsonic power of the blood is subject to considerable variation, but non-bacterial factors which depress it are but little known. Hektoen and Ruediger[2] have shown that a variety of inorganic salts inhibit the opsonic activity of the serum in the test-tube, but what effect similar substances have during life is not known.

Wright and Reid[3] further have shown that exudates produced by a given bacterium may exhibit partial or complete absence of opsonin for this microörganism. The fluid in the peritoneal cavity with tuberculous peritonitis may contain no opsonin for tubercle bacillus; it has, to use the expression of Wright and Reid, a low bacteriotropic tension.

The following experiment shows that an inflammatory exudate caused by one microörganism may contain no opsonin for this or for other microörganisms. A fatal quantity of Staphylococcus pyogenes aureus has been injected into the peritoneal cavity of the

[1] Received for Publication July 2, 1907.

[2] *Jour. of Infec. Diseases,* 1905, ii, 128.

[3] *Proc. of the Roy. Soc.,* 1906, lxxvii, 194.

guinea-pig and the power of the serum to opsonize Staphylococcus aureus and Bacillus typhi has been tested.

Experiment 1.—Into the peritoneal cavity of a guinea-pig were injected three cultures on agar-agar of Staphylococcus pyogenes aureus suspended in 3 c.c. of 0.85 per cent. sodium chloride. Death occurred in twenty-four hours; the peritoneal cavity contained 10 c.c. of turbid fluid from which clear serum was obtained by centrifugalization. The blood serum of a normal guinea-pig and 0.85 per cent. solution of sodium chloride were used for comparison. Since washed leucocytes of the guinea-pig are obtained with difficulty, corpuscles of the dog were employed. Bacteria used were suspended in 0.85 per cent. solution of sodium chloride. Each mixture contained equal volumes of its various ingredients. The following figures represent the average number of bacteria in a leucocyte after incubation at 38° C. during fifteen minutes.

With Staphylococcus pyogenes aureus:

Corpuscles + blood serum + staphylococci	16.0
Corpuscles + exuded serum + staphylococci	2.0
Corpuscles + salt solution + staphylococci	3.7

With Bacillus typhi:

Corpuscles + blood serum + B. typhi	8.44
Corpuscles + exuded serum + B. typhi	0.16
Corpuscles + salt solution + B. typhi	0.48

To determine if the serum of the exudate was capable of preventing phagocytosis in the presence of blood serum, phagocytosis in a mixture containing two volumes of normal blood serum was compared with phagocytosis in a similar mixture in which one volume of blood serum was replaced by a volume of exuded serum.

Corpuscles + blood serum + blood serum + staphylococci	9.2
Corpuscles + blood serum + exuded serum + staphylococci	11.5
Corpuscles + blood serum + blood serum + B. typhi	6.6
Corpuscles + blood serum + exuded serum + B. typhi	7.0

The experiment shows that opsonin for both Staphylococcus pyogenes aureus and for Bacillus typhi are absent in the exudate produced by intra-peritoneal injection of the first named micro-organism. The exudate thus produced (containing so-called aggressin) fails to inhibit phagocytosis when opsonin is supplied by addition of normal blood serum.

A second experiment with an exudate obtained by injecting the bacillus of swine plague into the pleural cavity of the rabbit gave a similar result. It is noteworthy that this organism, virulent for the

rabbit, failed to undergo phagocytosis in the presence of the blood serum of a normal rabbit, yet almost completely deprived the exudate of power to promote phagocytosis of Staphylococcus pyogenes aureus or of Bacillus dysenteriæ.

Experiment 2.—Into the right pleural cavity of a rabbit was injected one culture on agar-agar of Bacillus sui pestis; death occurred in about twenty hours. The exudate present in the pleural cavity was centrifugalized and tested with B. sui pestis, Staphylococcus pyogenes aureus and B. dysenteriæ. Blood serum of a normal rabbit and washed corpuscles of the dog were used. Each mixture contained four equal volumes as follows:

With Bacillus sui pestis:

Corpuscles + blood serum + salt solution + B. sui pestis..........	0
Corpuscles + exuded serum + salt solution + B. sui pestis........	0
Corpuscles + blood serum + exuded serum + B. sui pestis........	0.04

With Staphylococcus pyogenes aureus:

Corpuscles + blood serum + salt solution + staphylococci..........	10.4
Corpuscles + exuded serum + salt solution + staphylococci.......	2.2
Corpuscles + blood serum + exuded serum + staphylococci.........	22.7

With Bacillus dysenteriæ:

Corpuscles + blood serum + salt solution + B. dysenteriæ.........	2.4
Corpuscles + exuded serum + salt solution + B. dysenteriæ.......	0.0
Corpuscles + blood serum + exuded serum + B. dysenteriæ........	0.3

With one exception (B. dysenteriæ, Experiment 2) more active phagocytosis has occurred in the presence of a mixture of both blood serum and exudate than in blood serum alone, and this increased activity has been observed even when a mixture containing one volume of blood serum and one volume of exuded serum has been compared with a mixture containing two volumes of blood serum. The following experiment confirms this observation.

Experiment 3.—In the peritoneal cavity of a guinea-pig, twenty-four hours after injection of four agar-agar cultures of B. typhi, were found 10 c.c. of turbid fluid. The following tests were made after centrifugalization, the blood serum of a normal guinea-pig and the washed leucocytes of a dog being used.

With Bacillus typhi:

Corpuscles + blood serum + salt solution + B. typhi..............	14.0
Corpuscles + blood serum + exuded serum + B.typhi..............	21.2

With Staphylococcus pyogenes aureus:

Corpuscles + blood serum + salt solution + staphylococci	16.0
Corpuscles + blood serum + exuded serum + staphylococci	26.1

The exuded serum was not tested with bacteria alone until it had stood for some time and the results are not therefore comparable with the foregoing.

Corpuscles + exuded serum + B. typhi	0.2
Corpuscles + exuded serum + staphylococci	3.74

Evidence that absence of opsonic activity may bear no relation to bacterial injection is furnished by examination of the sterile pus obtained by repeatedly injecting small quantities of turpentine into the pleural cavity of the dog.

Experiment 4.—Serum of pus was obtained by centrifugalization of pus removed from the pleural cavity of the dog after repeated injection of turpentine. Absence of bacteria in the exudate was demonstrated by the negative result of attempted cultivation and by examination of stained preparations. The opsonic content of the fluid part of the pus was tested by the usual method, washed corpuscles of the dog and a suspension of Staphylococcus pyogenes aureus being used. For comparison, activity of phagocytosis in the presence of blood serum of the dog and in physiological (0.85 per cent.) salt solution was determined.

Corpuscles + blood serum + staphylococci	5.5
Corpuscles + exuded serum + staphylococci	0.5
Corpuscles + salt solution + staphylococci	0.4

The following experiment confirms that just described and shows, moreover, that diminution of phagocytosis is due to loss of opsonic action and not to the inhibiting action of turpentine.

Experiment 5.—An abscess was produced by injecting one cubic centimeter of turpentine into the subcutaneous tissue of the dog. Serum was obtained by centrifugalization from the thick sterile pus present at the end of four days. The opsonic activity of this serum was compared with that of the blood.

Corpuscles + blood serum + staphylococci	1.9
Corpuscles + exuded serum + staphylococci	0.5
Corpuscles + salt solution + staphylococci	0.1
Corpuscles + blood serum + blood serum + staphylococci	2.7
Corpuscles + blood serum + exuded serum + staphylococci	2.8
Corpuscles + exuded serum + exuded serum + staphylococci	1.2

Phagocytosis with serum of the exudate is much less than with blood serum, but when serum of the exudate is mixed with blood serum, phagocytosis is undiminished.

With the serum of the sero-fibrinous pleurisy caused by turpentine injected into the pleural cavity phagocytosis is approximately equal to that which occurs in the presence of blood serum. The following experiment is cited to show that turpentine does not necessarily destroy opsonic activity.

Experiment 6.—Serum from a sero-fibrinous exudate withdrawn from the pleural cavity of the dog three days after injection of 2 c.c. of turpentine was freed from cells by centrifugalization.

Corpuscles + blood serum + staphylococci	10.7
Corpuscles + exuded serum + staphylococci	11.1

The experiments show that the serum of a purulent exudate obtained after the cells have been deposited by centrifugalization may contain no opsonin for staphylococci, even though it has been produced by a sterile irritant and contains no microörganisms. This fact is in agreement with the observation made upon exudates caused by bacteria injected into the guinea-pig and rabbit, for here injection of one microörganism caused loss of opsonin for the other microörganisms which were tested. Nevertheless the experiments do not demonstrate absence of specificity on the part of opsonins, and in Experiment 3 a certain degree of specificity is perhaps suggested, injection of typhoid bacillus causing almost complete disappearance of opsonin for this microörganism, but only partial disappearance of opsonin for staphylococcus.

Absence of opsonin in the exudate produced by an inflammatory irritant of great activity has no necessary relation to the nature of the irritant, for in a purulent exudate caused by a sterile irritant there may be disappearance of opsonin for staphylococci. It is not improbable that the complement-like opsonin of normal sera, which, as Muir and Martin[4] have shown, is absorbed by a great many substances exhibiting combining affinities for complement, has been absorbed in the experiments just described not only by microörganisms but by cells contained in immense number in the purulent exudate, for it is well known that various tissue extracts can absorb complement.

The observations which have been described probably explain in part the presence of the innumerable extracellular bacteria which may be found in the serum of many purulent exudates. With a microörganism such as the meningococcus, streptococcus and staphylococcus, which readily undergoes phagocytosis in the body or *in vitro,* it is possible that exhaustion of opsonic content of the fluids, rather than impaired phagocytic power of the cells, may explain the occurrence of uningested microörganisms in considerable number in the exudate produced by these bacteria.

[4] *British Med. Jour.,* 1906, ii, 1783; *Proc. Roy Soc.,* 1907, B. lxxix, 187.

EXPERIMENTAL LIVER NECROSIS; I. THE HEXON BASES.[1]

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This communication is the first of a series of five, which presents the results of a somewhat comprehensive investigation of the chemical changes associated with experimental liver necrosis and which includes, in addition to that here presented, studies of the nitrogenous metabolism, nuclein metabolism, the activity of the intracellular hepatic enzymes and the changes in the fats and lipoids of the liver.

The advantage of undertaking such a comprehensive study was suggested by the investigations[2] which one of us had previously made of the necrosis caused in the liver of the dog by the intravenous or intraperitoneal injection of hæmagglutinative and hæmolytic immune sera. The lesions so produced are frequently focal and resemble in a general way those of eclampsia while the diffuse lesions, with the associated repair, are more or less similar to certain stages of acute yellow atrophy. It seemed plausible therefore that a study of the chemistry of such lesions, readily produced experimentally, might throw light not only on certain functions of the liver but might offer new knowledge of value in explaining

[1] Conducted under grants from the Rockefeller Institute for Medical Research. Read by title before the Society of Experimental Biology and Medicine, New York, March 20, 1907. Received for publication July 2, 1907.

[2] Pearce, R. M., The Experimental Production of Liver Necroses by the Intravenous Injection of Hæmagglutinins, *Jour. of Med. Research*, 1904, xii, 329; 1906, xiv, 541. Experimental Cirrhosis of the Liver, *Jour. of Exper. Med.*, 1906, viii, 64.

some of the problems of eclampsia, acute yellow atrophy and similar lesions in man.

The study of the hexon bases was undertaken in the hope of determining the character of the processes underlying the production of *intra vitam* autolysis. We have therefore attempted to determine the relative enzymotic power of the normal and the necrotic liver in regard to the synthesis or decomposition of the hexon bases. The importance of these bodies, as products of autolysis, has been emphasized by Wakeman[3] in his study of the livers of dogs poisoned with phosphorus or repeatedly anæsthetized with chloroform. In addition to the study of fresh tissues, we have investigated also the changes occurring in hexon nitrogen during antiseptic autolysis of liver substance.

The experimental lesions under consideration may be described, without going into detail, as hyaline necroses with little or no leucocytic reaction, the position and extent of which vary according to the amount of serum administered and the resistance of the animal. Small doses cause focal lesions more or less isolated and irregularly distributed; large doses produce a diffuse necrosis which spares only the tissue about the larger portal spaces. The lesions are found chiefly near the surface of the liver, but may occur in the deeper portions. In animals dying within a few hours after injection and before the appearance of necrosis an intense congestion of the entire portal system exists with, in the liver, innumerable thrombi composed of fused red cells.

In addition to such material from the dog, the livers of horses presenting degenerative and necrotic lesions, occurring in the course of immunization with various bacterial products, have also been utilized. These lesions, which have been described fully elsewhere by Pease and Pearce[4] and more recently by Lewis,[5] consist of widespread necrosis associated not infrequently with an extensive deposition of amyloid and usually accompanied by hæmorrhages.

[3] Wakeman, A. J., On the Hexon Bases of Liver Tissue under Normal and Certain Pathological Conditions, *Jour. of Exper. Med.*, 1905, vii, 292.

[4] Pease, H. D., and Pearce, R. M., Liver Necrosis and Venous Thrombosis in Horses Actively Immunized with Diphtheria and Tetanus Toxins and with Streptococci and their Products, *Journ. of Infect. Diseases,* 1906, iii, 619.

[5] Lewis, P. A., Hæmorrhagic Hepatitis in Antitoxin Horses, *Jour. of Med. Research,* 1906, xv, 449.

A series of fifteen livers, eleven from the dog and four from the horse, has been examined. Of the former, five were normal, four showed focal necroses and two diffuse necrosis; of the latter two were normal and two were examples of necrosis associated with extensive amyloid transformation.

Methods.—As the necrotic lesion in the dog's liver reaches its greatest extent in twenty-four to forty-eight hours after injection, all livers used in the study of the hexon bases were taken from animals killed at the end of the latter period. In the case of the horses the livers were removed within one hour after death.

After cutting away the larger vessels and the coarser tissue about the hilum the organ was subdivided and passed through the hashing machine. Small portions were weighed and set aside for drying and upon these total nitrogen estimations were made later. At the same time two portions of 100 grams each of moist liver were placed in flasks. To one, 600 cubic centimeters of water and 300 grams of sulphuric acid were added and hydrolysis carried on for fourteen hours in a paraffin bath. The other sample was suspended in 600 cubic centimeters of water and with it were thoroughly mixed about fifty cubic centimeters of toluol. This flask plugged with cotton was allowed to stand at room temperature for periods of one or two months. At the end of this time it was examined bacteriologically, and if found free of bacteria, 300 grams of sulphuric acid were added and the material hydrolyzed in the same manner as the control. Subsequent treatment was carried out according to the method outlined by Wakeman except that the process was continued only to the point of making nitrogen determinations of fractions "A" (arginin and histidin) and "B" (lysin) after removal of the barium and silver by means of sulphuric acid and hydrogen sulphide. For our purpose no advantage was to be gained by separating arginin and histidin.

Dry Solid Content.—An examination of the figures in the table indicates that the dry solid content of the dog livers with scattered focal necroses did not vary from the normal average of 24 per cent. In the case of the diffuse lesions, where the autolysis of the protoplasm was obviously more marked, the dry solids were reduced to an average of 20.8 per cent. The figures referring to normal livers

TABLE—*Hexon Bases.*

Dog.			Determination on Fresh Tissue. Per Cent. Nitrogen in Dry Substance.	Per Cent. N_2 ppt. by Phospho-tung. Acid.	* Per Cent. Nitrogen as Arginin and Histidin.	* Per Cent. Nitrogen as Lysin.	* Per Cent. Nitrogen as Purin Bases.	On Autolysed Tissue. Per Cent. of N_2 ppt. by Phospho-tung. Acid.	* Per Cent. Nitrogen as Arginin and Histidin.	* Per Cent. Nitrogen as Lysin.	* Per Cent. Nitrogen as Purin Bases.
3			12.1	13.2	4.3 32.5	3.6 27.1	5.3 40.4	Not bacteria-free			
4			10.9	15.0	6.0 40.0	4.9 32.7	4.1 27.3	19.8	8.4 42.4	9.8 49.5	1.6 8.1
13			11.8	Used only for autolysis				18.3	8.0 43.7	7.3 39.9	3.0 16.4
14			9.2	Used only for autolysis				17.6	7.5 42.6	7.6 48.2	2.5 14.2
17			11.2	19.6	4.3 36.2	7.4 37.7	5.1 26.1	20.4	8.0 39.2	7.9 38.7	4.5 22.1
Av.			11.3	15.9	6.0 36.2	5.3 32.5	4.8 31.3	19.0	8.0 42.0	8.2 42.8	2.9 15.2
5			21.4	12.2	5.6 45.8	4.2 34.4	2.4 19.8	9.0	4.1 45.5	2.7 30.0	2.0 24.5
7			19.1	14.5	7.3 50.2	5.1 35.1	2.1 14.7	10.2	4.9 48.0	3.5 34.3	1.9 17.7
9			22.8	10.2	4.8 47.2	3.2 31.5	2.1 21.3	6.7	2.3 34.3	2.6 38.8	1.8 26.9
10			23.7	8.5	3.6 42.3	2.5 29.4	2.4 28.3	6.8	2.7 39.8	1.9 27.9	2.1 32.3
Av.			21.8	11.4	5.3 46.4	3.8 32.6	2.2 21.0	8.2	3.5 41.9	2.7 32.8	2.0 25.3
29	Extensive diffuse necrosis	21.9	12.7	30.0	15.6 52.0	11.6 38.6	2.8 9.4	23.9	11.5 48.1	10.2 42.7	2.2 9.2
60	Extensive diffuse necrosis	19.8	13.2	25.6	12.7 49.6	11.3 44.1	1.6 5.3	20.7	10.1 48.8	9.5 45.9	1.1 5.3
Av.		20.8	12.95	27.8	14.2 50.8	11.5 41.4	2.2 7.4	22.3	10.8 48.5	9.9 44.3	1.7 7.3

TABLE—*Hexon Bases* (*Continued*).

Nature of Experiment.		Determination on Fresh Tissue.					
		Per Cent.			* Per Cent. Nitrogen as		
Horse.	Condition of Liver.	Dry Substance.	Nitrogen in Dry Substance.	N_2 ppt. by Phosphotungstic Acid.	Arginin and Histidin.	Lysin.	Purin Bases.
60	Normal	23.3	12.0	8.1	3.0 / 37.0	4.6 / 56.7	0.5 / 6.3
65	Normal	21.8	11.2	8.5	3.9 / 45.9	3.1 / 36.5	1.0 / 17.6
Av.		22.6	11.6	8.3	3.5 / 41.5	3.9 / 46.6	0.75 / 12.0
42	Necrosis and amyloid	20.0	21.7	13.5	2.9 / 21.4	8.2 / 60.7	2.4 / 17.9
69	Necrosis and amyloid	21.4	13.7	13.3	1.2 / 9.0	10.2 / 76.7	1.9 / 14.3
Av.		20.7	17.7	13.4	2.1 / 15.2	9.2 / 68.7	2.2 / 16.1

* Figures in upper left hand corner are calculated on the total nitrogen; those in the lower right hand corner on the total hexon nitrogen.

agree somewhat closely with those of Wakeman, who, however, noticed a slight decrease in the percentage of dry substance in the livers of dogs poisoned by phosphorus. This is somewhat surprising since he describes these livers as markedly fatty. If the protoplasm of which seventy-five per cent. is water is replaced by fat, which contains none, the dry solids should increase instead of decrease. If Wakeman's figures are correct they show a marked increase in the water content of the organs during phosphorus poisoning. That such an increase may occur did not appear in our investigation[6] of fatty changes in the liver.

Total Nitrogen Content.—Several interesting facts developed from the analysis of the nitrogen of the dry solids. The average of the five determinations for normal tissue was 11.3 per cent. in agreement with Wakeman; while that of the livers with lesions of a scattered focal character was 21.8 per cent.

This, of course, indicates a deposition or heaping-up of nitrogenous material in the hepatic cell. That such a process may take place even under physiological conditions is evident from the results of experiments carried out by Seitz.[7] This investigator found that by feeding hens and geese excessive amounts of meat a true deposition of nitrogenous substances occurred in the cells of the liver. This increase amounted in some instances to 300 per cent.

In our experiments the quantity of nitrogen in the hepatic cell was almost doubled in the organs with scattered necrotic lesions. This condition allows of an explanation similar to that offered for the infiltration of fat in tissues during phosphorus or phloridzin poisoning. In such lesions the cells have lost in part their power to oxidize properly the sugar or other materials placed at their disposal by the circulating blood and hence the starving cell, in its endeavor to spare its own protoplasm from destruction, stores up fat for purposes of oxidization.

That the cells at the margin of the necroses under consideration do accumulate fat we have observed in our study of the histological changes occurring in these livers.[6] It seems distinctly possible that

[6] See fifth paper of this series, "The Fats and Lipoids" in this number of the *Journal*.

[7] Seitz, W., Die Leber als Vorrathskammer für Eiweissstoffe, *Arch. f. ges. Physiol.*, 1906, cxi, 309.

during the initial stage of congestion and thrombosis and in the early stages of necrosis, the imperfectly nourished and slightly injured cells may heap up nitrogenous material also. When, however, the lesion is more extensive, the storing up of nitrogen is not so evident as shown by the fact that the nitrogen of the dry solids remains more nearly normal. This is to be explained by assuming that the nitrogen stored up in the persisting liver cells is sufficient to more than balance the loss by autolysis in the necrotic areas.

Wakeman's figures for the nitrogen content of livers after phosphorus poisoning show a diminution equivalent to 35.6 per cent. and a corresponding decrease in the hexon base nitrogen. This indicates, according to his view, that that part of the proteid molecule involving the hexon bases has not undergone a relatively greater decomposition than the other nitrogenous substances. It would seem to us that the low nitrogen content of the phosphorus livers is wholly, or in greatest part, due to the large amount of fat present.

The Hexon Bases.—Wakeman's results indicate that in the dog the average nitrogen content of the bases in the normal liver tissue is 17.04 per cent. of the total nitrogen, while in the liver of dogs poisoned with phosphorus it is only 10.72, a falling off of 37.1 per cent. The livers of dogs receiving chloroform showed 13.6 per cent., a decrease of 20 per cent. In these figures Wakeman sees evidence of increased autolysis in hepatic cells affected by phosphorus or chloroform. Although he mentions definite necrosis in but one of his livers, the cell destruction in phosphorus poisoning, gradual as it is, is such that our results ought to fall, as they do, somewhat into line with his.

Thus as an average of three normal livers we find the figures concerning the total content of hexon bases, based on nitrogen content attributable to them, to be 15.9 per cent. of the total nitrogen. In the case of the scattered focal necrosis, the percentage is slightly decreased to 11.4 as an average of four determinations, but the absolute amount is increased. That is gram for gram of dry substance there occurs an increase in the absolute amount of hexon bases which however appears as a decrease in percentage on account of the high nitrogen content of the dry substance.

This absolute increase in hexon content of dry substance is greater and more clearly accentuated in the livers of those dogs in which the necrosis is more diffuse. Here the nitrogen of the dry substance is almost the same as that of the normal, 12.95 per cent. as average of two determinations; but the hexon base nitrogen content rises to 27.8 per cent. of the nitrogen of the dry substance.

This observation is extremely interesting in that it points most strongly to the preponderance of the autolytic process over the synthetic in the more widespread forms of necrosis with early repair. The figures show a definite increase in the hexon base content of the necrotic cell, although the accumulation of nitrogen in this lesion, 12.95 per cent., could not occur to such a marked extent as it did in the focal lesion, 21.8 per cent., because of the lessened number of persisting living cells capable of storing up nitrogen. A rearrangement of nitrogen, the result of autolysis in the larger areas of necrosis, therefore took place as shown by the hexon nitrogen content of 27.8 per cent., as compared with that of 11.44 per cent. in the focal lesion and 15.9 per cent. in the normal. This great increase of hexon bases may be due in part also to disturbances of the circulation accompanying the necrosis which prevent the diffusion and removal of the bases from the liver. In this connection attention may be called to Jacoby's[8] observation that leucin and tyrosin are not found in the liver of phosphorus poisoning when no disturbance of the hepatic circulation exists.

Relation of Precipitate "A" (Arginin and Histidin) and Precipitate "B" (Lysin) to the Total Hexons.—Wakeman, from a consideration of his results on these fractions, concludes that in the autolysis which occurs in the cell in phosphorus poisoning the arginin suffers a greater destruction than do the other bases, probably through the action of arginase, which splits arginin into ornithin and urea.[9] His tables show that of the 17.0 per cent. of the nitrogen of the total bases in the normal tissues, 11.8 per cent. is to be attributed to arginin and histidin and 5.2 to lysin. In phosphorus poisoning, on the other hand, the nitrogen of the bases amounts to

[8] Jacoby, M., Ueber die Beziehungen der Leber und Blutveränderungen bei Phosphorvergiftung zur Autolyse, *Zeit. f. physiol. Chem.*, 1900, xxx, 174.

[9] Kossel, A., and Dakin, H. D., Ueber die Arginase, *Zeit. f. physiol. Chem.*, 1904, xli, 321.

10.7 per cent., of which only 6.8 per cent. belongs to the arginin and histidin and 3.8 per cent. to the lysin. This indicates a decrease during autolysis of 42.3 per cent. for the arginin and histidin, but only 26.8 per cent. for the lysin.

If, however, one considers these figures from the standpoint of the relationship which the precipitates "A" and "B" bear to the total hexon bases of the normal and phosphorus dogs, an entirely different view is obtained. In the normal tissue precipitate "A" (arginin and histidin) forms 69.6 per cent. and precipitate "B" (lysin) 30.4 per cent. of the total bases; whereas in the phosphorus livers the former is 63.9 per cent. and the latter 36.1 per cent. Hence the decrease in the fraction "A" is only 5.7 per cent. and this is offset by the corresponding increase in fraction "B." This diminution in the arginin and histidin content of the hexon base fraction of the livers of phosphorus-poisoned animals compared with the normal is so slight that it hardly seems warrantable to attribute it to the action of arginase.

Our figures for the total hexon bases (15.9 per cent.) in the normal agree well with those found by Wakeman, while those for the focal necroses have suffered a percentage decrease which is somewhat comparable to that noticed by him in phosphorus poisoning. Wakeman's percentage decrease, however, was also an absolute one while ours in reality was an absolute increase, as has been explained above. The proportion which precipitates "A" and "B" bear to the total is markedly different, however, from that which he notes. Our average in the normal tissues for the arginin and histidin fraction is 36.2 per cent. as against 69.4; the lysin fraction 32.5 per cent. more nearly agrees with his 30.3 per cent. If we exclude the purin bases our results become more comparable and agree better in percentage.

We have also found a much greater percentage of the total nitrogen due to purin bases. Wakeman's figures show an average of 0.0273 per cent. for normal and pathological, while ours showed 4.8 per cent. for the normal tissue against 2.2 per cent. for the focal necrotic lesions and the same for the diffuse necrosis.

As to variations which the individual bases undergo in their relation to the total bases, our results point to an increase from the

normal of 36.2 per cent. to 46.4 during the focal necrosis and to 50.8 per cent. during diffuse necrosis for arginin and histidin. The lysin fraction shows no change in the focal necroses as compared with the normal, but in the diffuse necrosis it increased 24.3 over the normal in agreement with Wakeman.

In the autolysis *in vitro,* which is discussed in the next paragraph, the normal fraction " A " represented 42.0 per cent. of the whole bases and fraction " B " 42.8 per cent.—differences from the normal which are well within the limit of error. Hence the absolute increase from 6.0 and 5.3 in the unautolyzed to 8.0 and 8.2 per cent. of the total nitrogen in the tissue after autolysis was in exact relation to the increase in total hexon nitrogen. The same is true for the autolyzed tissue with necrosis of all types.

This would emphasize more markedly the point made above that small evidence can be adduced to show that an enzyme, arginase, is acting on the arginin, decreasing its amount during autolysis. Such action is not shown by our figures and moreover in our investigation of intracellular hepatic enzymes[10] we could not obtain an active arginase from the necrotic dog's liver, though it was found in the normal.

Hexons Resulting from Autolysis in Vitro.—It seemed worth while in view of the investigation, carried on synchronously, of intracellular hepatic enzymes[10] to determine the relation of the hexon bases to autolysis of the liver *in vitro.* For such observations the figures given above for autolysis during life serve as controls. Autolysis was allowed to proceed for varying lengths of time in the endeavor to determine whether the different organs showed varied degrees of autolysis. The periods selected, one and two months, were inadequate to bring out this point, since the autolysis was completed or the reaction reached its equilibrium before one month. This was unfortunate as we thereby disregarded the important element, that of time, in this connection. The time element, however, is fully considered elsewhere[10] from another point of view.

A glance at the figures in the table shows that after autolysis of the normal organs the percentage of total nitrogen as hexon

[10] See second paper of this series, " Enzymes," in this number of the Journal.

nitrogen was 19.0 per cent. as an average of four determinations. This increase of 18.8 per cent. over the normal hexon content is not marked, and if one examines the figures referring to the two dogs (4 and 17) upon which alone we have absolute controls, it will be seen that this increase is variable.

We are not inclined to attempt to explain this result in detail in this place, since the data which we will present in our study of the enzymes bear more decisively upon this matter. Suffice it to say that these figures, taken in connection with those of the diffusely necrotic organs where an increase also was evident, indicate that in the autolysis a transformation or rearrangement occurs by which nitrogenous atomic complexes, not normally yielding hexon bases, become altered into hexon bases or their combinations.

In all degrees of necrosis, the autolyzed material contained a smaller amount of hexon bases than the unautolyzed. In the liver with scattered focal necroses the decrease of the hexon nitrogen in per cent. of the total nitrogen amounted to 28.0 per cent.; in the diffuse necrosis 19.8 per cent. This would seem to imply that in the living tissue the hexon splitting enzyme is to some degree inhibited, probably through the action of the blood serum.

Hexon Bases in the Liver of the Horse.—The results obtained with the normal livers of horses agree in regard to the dry solids (22.6 per cent.) and the nitrogen of the dry tissue (11.6 per cent.) with those obtained for the dog. The nitrogen precipitable with phosphotungstic acid, however, is surprisingly low, amounting to only 8.3 per cent. It would seem inadvisable to attempt an explanation of this difference, since these animals were not absolutely normal, in that they had been utilized for the purpose of preparing antitoxin and had died during such treatment, though no lesions were found in the liver. The injection of bacterial products may set up processes in the cell which tend to reduce its hexon content without changes evident histologically. The percentage which fractions "A" and "B" of such livers bears to the total hexon content is not far removed from that found for each in the case of the normal dog liver.

The two lesions which served as examples of necrosis presented the complicating feature of amyloid. This fact renders the series

not exactly comparable to the previous one. The increase in nitrogen of the dry substance over that of the normal is present here also, and the total hexon base nitrogen has increased to 13.4 per cent. This latter result may be caused by the heaping up of the bases as products of autolysis in the large necrotic areas. More probably, however, it is to be attributed to the amyloid degeneration, in which the normal cellular proteids with relatively small percentage (10–30 per cent.) of diamino-nitrogen are replaced by amyloid, the proteid constituent of which, in the liver and spleen as shown by Neuberg,[11] contains twice as much (50–60 per cent.) diamino-acid nitrogen. This rearrangement in the hexon content of the proteids of the organ has also resulted in a marked change in the relation which the arginin and histidin as well as the lysin bears to the total amount of hexon base precipitate. "A" forms only 15.2 per cent. and precipitate "B" 68.7 per cent. of the whole diamino-acid nitrogen.

SUMMARY.

1. The liver of the dog in which necrosis has been produced by injection of hæmatoxic immune sera is characterized in the less marked forms by a storing up of nitrogen in the persisting living cells, while in the diffuse forms the total nitrogen content is but slightly above the normal. This last is to be explained by the great diminution in persisting liver substance which limits the power of nitrogen accumulation.

2. In all forms of necrosis there occurs an absolute increase of nitrogen precipitable by phosphotungstic acid (hexon bases) but the percentage increase, in relation to total nitrogen, diminishes in those forms (focal) in which the products of autolysis may be readily carried off by the blood stream and greatly increases in the diffuse form with large areas in which the circulation is seriously impaired.

3. Although the absolute amount of nitrogen representing arginin and histidin varies, a relative increase is evident when this fraction is compared with the total diamino-nitrogen. This increase corresponds to the degree of necrosis and attendant circu-

[11] Neuberg, Ueber Amyloid, *Verhand. d. Deut. path. Gesellsch.*, 1904, vii, 19.

latory disturbance and indicates that in necrosis as opposed to degeneration (Wakeman) arginin is not split up by arginase. The lysin also bears a definite relation to the total hexon nitrogen.

4. The diamino-nitrogen of the normal liver after autolysis *in vitro* shows a slight variable increase over that of the unautolyzed, while the necrotic livers showed a decided decrease.

5. The diamino-acid nitrogen of normal horse liver is only about one half of that of the dog; the relative proportion of the bases is about the same. In necrotic livers with amyloid the diamino-nitrogen is markedly increased which is in accord with Neuberg's observations on the high hexon base content of amyloid.

Conclusions.—Upon the whole then the chemical processes occurring in the hepatic cell undergoing rapid or immediate necrosis and those accompanying a slow "degeneration," as for example in phosphorus poisoning, must be different and distinct as would be expected from the histological findings. In necrosis we find the cell in a complete state of disorganization and decomposition and hence autolysis begins immediately, but in the changes occurring in the cell in the so-called degenerations, as phosphorus poisoning, the nucleus remains intact, thereby insuring to a certain extent the life or at least partial function of the cell. That under the latter circumstance a disturbed condition does exist is evidenced by the heaping up of fat in the cell, and although the results of the various investigations upon the altered processes in the liver of animals poisoned with phosphorus tend to show that this change is an autolysis, in which certain amino-acids appear as the result of the splitting of the proteid molecule, it is not of the same type as that appearing in the necrotic cell. This is shown by a comparison of Wakeman's findings, which indicate definitely a diminution of hexons in the liver, with ours which show a great increase.

Examinations of the human liver, by a direct method without hydrolysis, but few in number, it is true, tend to the same conclusion. Taylor,[12] for example, found arginin in a liver with wide-

[12] Taylor, A. E., Ueber das Vorkommen von Spaltungsprodukten der Eiweisskörper in der degenerirten Leber, *Zeit. f. physiol. Chem.*, 1902, xxxiv, 580. On the Occurrence of Amino-acids in Degenerated Tissue, *Univ. of California Publications*, 1904, i (Path.), 43.

spread necrosis, the result apparently of chloroform poisoning; from seven other livers, representing various lesions (dysenteric abscess, fatty degeneration, pyæmia and acute yellow atrophy), he was unable to isolate this substance. Soetbeer[13] also was unable to find hexon bases in a peculiar type of cirrhosis with acute degeneration.

This difference in the hexon content of the liver of "degeneration" and that of necrosis is so striking that it would appear to be due to a difference in the nature or rapidity of the cell destruction, though it may to some extent be explained by the disturbances of circulation which occur in the necrosis and which, presumably, are absent in degeneration.

Our observations show also that care must be exercised in drawing conclusions from results obtained by autolysis *in vitro*. It seems distinctly doubtful whether the autolysis of the cell which occurs under such circumstances has any relation to autolytic changes during life.

[13] Soetbeer, F., Ueber einen Fall von akuten Degeneration des Leberparenchyms, *Arch. f. exper. Path. u. Pharm.*, 1903, l, 294.

EXPERIMENTAL LIVER NECROSIS; II. ENZYMES.[1]

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The experiments about to be described represent an attempt to determine the relation of the intracellular hepatic enzymes to chemical changes occurring in liver necrosis. Our results are based on a comparison of the variations in the enzymotic equilibrium of the normal hepatic cells with those occurring in necrosis of varying grades of severity. At present the chief and most promising method of detecting such variations consists in determining by means of post-mortem autolysis the condition under which the cell is existing at the time of the death of the animal, and the rapidity, nature and extent of the changes which occur after the commencement of the autolysis. We are well aware that the interpretation of the results of post-mortem autolysis in relation to cellular activity during life is open to objection and may not have the importance usually ascribed to it.

Our investigation of the enzymotic activity of the liver tissue under normal circumstances and in varying degrees of necrosis may naturally be subdivided as follows:

1. A determination in a quantitative way of the degree of autolysis which the tissue undergoes after death.

2. A study of the individual enzymes with reference to the part which they play in the general course of autolysis.

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3. A determination of the products formed as the result of such autolysis. These include the diamino-acids, which have been considered in the preceding paper,[2] where they more properly belong, and the monamino-acids to which, as represented by leucin and tyrosin, we have given considerable attention. It was our intention to determine, by perfusion of livers in various stages of necrosis, the changes in the composition of the blood which might occur, but owing to the great amount of labor entailed in the present studies this has been unavoidably postponed.

Comparative Estimation of Products of Autolysis.—In the study of the changes which the nitrogenous material undergoes during autolysis *in vitro* an attempt was made to carry out a partition analysis of the non-coagulable nitrogen. This method has already been employed with good results by v. Drjewezki[3] to determine the effect of alkalies of varying strengths upon autolysis. His results, as well as those of Wiener,[4] point to the sensitiveness of the autolytic enzymes to changes in reaction, especially those due to alkalies, and these investigators conclude that the alkalies of the serum are responsible for the well-known inhibitory effect of the serum upon autolysis. Baer[5] and his associate, Loeb,[6] admit the inhibitory effect of the serum but are inclined to attribute it to the action of the serum globulin.

These facts, as well as those brought out by Lang[7] concerning the inhibitory effect of large quantities of toluol upon autolytic processes, although other factors may have influenced the results of the latter, all tend to emphasize the fact that in performing experiments of this character too much attention cannot be given

[2] See first paper of this series, "Hexon Bases" in this number of the *Journal.*

[3] v. Drjewezki, A., Ueber den Einfluss der alkalischen Reaktion auf die autolytischen Vorgänge in der Leber, *Biochem. Zeit.*, 1906, i, 229.

[4] Wiener, H., Ueber den Einfluss der Reaktion auf autolytische Vorgänge, *Zent. f. Physiol.*, 1905, xix, 349.

[5] Baer, J., Ueber die Wirkung des Serums auf die intracellularen Ferments, *Arch. f. exper. Path. u. Pharm.*, 1906, lvi, 68.

[6] Baer, J. and Loeb, A., Ueber die Bedingungen der autolytischen Eiweissspaltung in der Leber, *Arch. f. exper. Path. u. Pharm.*, 1905, liii, 1.

[7] Lang, S., Ueber desamidierung im Tierkörper, *Beit. z. chem. Physiol. u. Path.*, 1904, v, 321.

to the attainment of absolutely comparable conditions in all the various experiments. With these points in mind we have endeavored to control our work in every way, as is shown in the following detail of the experiments.

Quantities of the fresh tissue of known weight were ground to such a state of subdivision that when mixed with water or neutral Ringer's solution the mixture could be readily drawn up in a pipette. This mixture, usually consisting of two hundred grams of liver, was made up to 1,200 cubic centimeters and placed in a sterile flask and the mixture covered with a layer of toluol. This latter substance was well shaken in, after which were pipetted off, as controls, two samples of two hundred cubic centimeters each. Both were thoroughly sterilized in the autoclave in order to stop autolysis; one was examined immediately, as an initial control, the other was placed with the original mixture in the thermostat (37.5° C.) and examined at the conclusion of the experiment as a final control. The material in the thermostat was shaken from time to time and at intervals of one, three, five and eight days samples of the main mixture were removed for analysis by the same method as the controls. The analysis of the samples, after they were shown to be bacteria free,[8] took place in the following manner:

The mixture was pipetted into a beaker and sufficient water was added to allow of easy coagulation of the proteid material present. Acetic acid was added to slightly acid reaction after the boiling point was reached. The coagulated proteid was removed by filtration and repeatedly and thoroughly washed with boiling water. The volume of the filtrate and washings was made up to eight hundred cubic centimeters. Of this, twenty-five cubic centimeters served for the determination of the total nitrogen by the Kjeldahl-Gunning method, one hundred for the estimation of ammonia according to the method of Shaffer as applied to the urine, one hundred for the uric acid determination, using the Hopkins-Folin method, and fifty to determine the amount of nitrogen not precipitable by phosphotungstic acid in sulphuric acid solution, the so-called

[8] For this purpose it was deemed sufficient merely to examine stained films though when the final sample of each mixture was taken cultures were made. In this series representing nine livers no contamination occurred.

monamino-nitrogen. An attempt was also made to determine the nitrogen precipitable (proteoses) by zinc sulphate but our results are so incomplete that little can be gained from their discussion. In all cases duplicate determinations were made and the figures given represent their average. Dogs were employed in all experiments.

In Table I are presented the results of the nine experiments which differed in their conditions for the purposes of control, as follows:

Two normal livers (52 and 54) with their usual blood content, the diluting fluid of one being distilled water and of the other neutral Ringer's solution. This solution was prepared in the ordinary way with the exception that the sodium bicarbonate was not added in order to avoid an alkaline medium.

Two normal livers (53 and 58) washed *in situ*, the one with water and the other with neutral Ringer's solution.

One necrotic liver four hours after injection (57). An attempt was made to wash this liver with water but on account of the extensive thrombosis it was only partly successful. It is therefore referred to as "half-washed."

Two necrotic livers forty-eight hours after injection (48 and 56); one unwashed diluted with water; the other washed and diluted with neutral Ringer's solution.

Two livers (43 and 49) five days after injection, both showing necrotic lesions with early repair; one washed and diluted with Ringer's, the other unwashed but diluted with water.

In each instance in which the livers were washed the procedure was begun under ether while the animal was alive. With the exception noted the livers were completely blanched save for slightly tinged areas about the more diffuse foci of necrosis.

The results in Table I, in terms of nitrogen, are expressed in percentages of the total nitrogen of the dry tissue and of the total non-coagulable nitrogen. A critical consideration of the figures presented allows of the following statements:

Non-coagulable Nitrogen.—The inhibiting effect of the blood serum upon the extent of the autolysis of both normal and necrotic tissue is decisively shown. The percentage of non-coagulable nitrogen in the case of the unwashed normal organs increased from 10.7 and 9.7 to 19.9 and 29.1 per cent. respectively, an increase of 100 and 200 per cent., on the eighth day; while in the washed normal livers the average increase at the eighth day amounted to 450 per cent. The increase in the five day necrotic unwashed liver was 127 per cent.; that of the washed tissue 349 per cent. The forty-

TABLE I.

Autolysis; nitrogen partition.

Normal.				4 Hours.	48 Hours. Necrosis.		5 Days. Necrosis.		Duration.
Not Washed.		Washed.		Washed.*	Not Washed.	Washed.	Not Washed.	Washed.	
52†	54	53	58	57	48†	56	43†	49	
Percentage of total nitrogen in non-coagulable form.									
10.7	9.7	13.5	9.5	8.5	12.7	8.3	26.6	18.3	Control
15.5	18.6	35.5	23.6	19.3	20.0	18.5	39.4	41.1	1 day
18.2	27.8	57.6	38.5	24.1	24.9	65.1	51.1	48.5	3 days
19.8	27.8	67.5	49.7	29.6	30.8	75.7	54.3	61.0	5 days
19.9	29.1	70.1	54.2	37.9	32.1	79.4	60.4	82.3	8 days
13.4	11.6	14.0	8.9	7.4	14.5	7.2	22.1	18.1	Final Control
Phosphotungstate-filtrate nitrogen (monamino-acid).									
7.2 / 67.3	6.7 / 69.3	7.4 / 54.8	4.2 / 44.5	5.5 / 64.7	10.7 / 81.1	3.6 / 43.0	15.0 / 56.5	11.3 / 51.7	Control
12.4 / 80.0	13.7 / 73.6	30.9 / 87.0	18.2 / 77.1	14.7 / 72.8	17.2 / 86.0	16.4 / 88.6	29.7 / 75.4	30.0 / 73.1	1 day
14.1 / 77.5	21.4 / 77.0	49.8 / 86.6	31.8 / 82.6	19.9 / 82.5	22.6 / 90.8	54.7 / 84.2	37.2 / 72.8	39.3 / 81.0	3 days
16.2 / 81.8	22.9 / 82.4	56.9 / 84.3	40.3 / 81.0	25.3 / 85.4	26.0 / 84.4	64.3 / 84.9	43.8 / 80.7	53.0 / 86.9	5 days
16.9 / 84.9	23.9 / 82.7	57.8 / 80.1	46.2 / 85.2	32.3 / 85.2	27.2 / 84.7	70.1 / 88.3	46.9 / 77.6	71.9 / 87.3	8 days
8.4 / 63.0	8.1 / 70.0	7.6 / 54.1	4.8 / 54.3	6.8 / 90.5	8.9 / 75.4	3.7 / 51.5	11.9 / 53.8	11.9 / 66.0	Final Control
Ammonia nitrogen.									
0.74 / 6.9	0.49 / 5.1	0.59 / 4.3		0.79 / 9.4	0.80 / 6.3	0.85 / 10.2	2.08 / 8.6	1.10 / 6.0	Control
1.38 / 8.8	0.90 / 4.8	1.41 / 4.0		1.05 / 5.4	1.46 / 7.3	1.50 / 8.1	4.4 / 10.6	2.20 / 5.3	1 day
1.45 / 7.9	1.06 / 3.8	2.45 / 4.2		1.9 / 7.9	2.16 / 8.6	2.78 / 4.3	4.7 / 9.2	3.30 / 6.9	3 days
1.63 / 8.2	1.60 / 5.1	2.52 / 3.8		1.6 / 5.4	2.19 / 7.1	3.25 / 4.3	5.8 / 9.6	3.99 / 6.5	5 days
2.08 / 10.4	1.22 / 4.2	2.45 / 3.5		2.9 / 7.6	2.34 / 7.3	3.01 / 3.8	6.4 / 10.6	5.11 / 6.2	8 days
0.66 / 4.9		0.67 / 5.2			1.02 / 8.6	0.85 / 11.6	2.2 / 10.0	1.43 / 7.9	Final Control

* Washing incomplete.

† Distilled water used instead of neutral Ringer's solution.

Figures in upper left-hand corner show percentage of total nitrogen; those in lower right-hand corner, of non-coagulable nitrogen.

eight hour washed tissue (Dog 56) with a very extensive necrosis, showed the greatest increase, equivalent to 856 per cent. of the control. The increase of the four hour experiment (congestion and thrombosis) hardly equaled that of the washed normal.

Wherever water was employed in washing or in diluting, the autolysis was distinctly less than when neutral Ringer was used. (Compare Dogs 52 and 54.)

Concerning the rapidity of the autolysis, it may be noticed that, though the initial increase during the first day in the case of the unwashed tissues is but one half of that of the washed, it represents, as does also the increase of the washed, fifty per cent. of the total autolysis. On the third day, however, the autolysis has reached its maximum in the unwashed tissues, while the washed organs continue to increase until the eighth day, when the autolysis in their case is also apparently complete.

In the forty-eight hour lesions in which, histologically, the autolysis of the necrotic areas would appear to be at its height, we see that the autolytic processes *in vitro* were also very active. The increase at the end of the eighth day in the unwashed liver (Dog 48) was about 150 per cent., but after the removal of the inhibitory action of the blood (Dog 56) the increase rose to almost 900 per cent. The same thing is evident in the fifth day lesions but is not so pronounced.

The rapidity with which the autolysis reaches its maximum is of course dependent upon various factors. The attainment of the maximum signifies that the reaction velocities of the system, made up of substrat, hydrolytic agent and enzyme, have reached an equilibrium, caused, no doubt, by the non-removal of the products of autolysis. Since we must assume that the substrat and enzyme are the same in the normal tissues of both washed and unwashed organs, the varying factor must consist in the hydrolysis which, from the work of Wiener, seems undoubtedly due to the unneutralized acids formed during autolysis as first described by Magnus-Levy.[9] The acids which are formed in the normal metabolism of the cells are neutralized by the ammonia and excess of bases in the blood; hence

[9] Magnus-Levy, A., Ueber die Säurehildung bei der Autolyse der Leber, *Beit. z. chem. Physiol. u. Path.*, 1902, ii, 261.

autolysis does not occur in the living cell. As soon as the serum with its neutralizing power is removed, as in the washed organs or where the acids use up the excess of bases as in the center of a large area of necrosis, the conditions necessary for autolysis are present and hydrolysis of the substrat protoplasm takes place.

Phosphotungstate Filtrate Nitrogen (*Monamino-acids*).—The phosphotungstate precipitate has been disregarded here, for, as it consists of diamino-nitrogen it has been sufficiently covered in the autolysis experiments in connection with the study of the hexon bases.[10]

By far the major portion of the nitrogen in the filtrate is in the form of monamino-acids.[11] The table indicates the percentage of the fraction in terms of the total nitrogen of the tissue as well as of the total non-coagulable nitrogen. Our figures for the controls indicate that in the normal tissue, washed or unwashed, 4.2 to 7.4 per cent. of the total nitrogen is to be attributed to nitrogen not precipitable with phosphotungstic acid. Of the forty-eight hour lesions, that with the most marked diffuse necrosis (Dog 48) showed 10.7 per cent. of the total nitrogen in that form, while the other, of the focal type (Dog 56), had only 3.6 per cent. or slightly less than the lowest of the normal figures. Also, in the first of this pair, 81.1 per cent. of the non-coagulable nitrogen was in the form of monamino-acid while the other showed only 43.0, again somewhat less than normal.

These two experiments illustrate most decisively the point which Taylor's[12] results seem to indicate. That is, there is an absence of monamino-acids in pathological conditions of the liver accompanied by little or no necrosis, while in necrosis of the diffuse type both the monamino- and diamino-acids are present. We have elsewhere[13] suggested that the relation of circulatory disturbances to

[10] See first paper of this series, "Hexon Bases" in this number of the *Journal.*

[11] v. Drjewezki, A., Ueber den Einfluss der alkalischen Reaktion auf die autolytischen Vorgänge in der Leber, *Biochem. Zeit.*, 1906, i, 229.

[12] Taylor, A. E., Ueber das Vorkommen von Spaltungsprodukten der Eiweisskorper in der degenerirten Leber, *Zeit. f. physiol. Chem.*, 1902, xxxiv, 580. On the Occurrence of Amino-acids in Degenerated Tissue, *Univ. of California Publications*, 1904, i (Path.), 43.

[13] See first paper of this series, "Hexon Bases" in this number of the *Journal.*

the removal of the products of autolysis is an all-important factor. This is further supported by the two experiments under discussion which indicate that the organ with the focal lesions contained no more monamino-nitrogen than did the normal tissue. In this case the circulation was very slightly, if at all, impaired, and these acids, if they were formed, were removed immediately by the blood stream. In Experiment 48 the large necrotic areas, the centers of which were remote from circulatory fluids, held the acids as they were produced. That these substances are produced in autolysis of this type *in vivo* in large quantities is also indicated by the fact that 81.1 per cent. of the non-coagulable nitrogen of this liver was present in the fresh tissue as nitrogen non-precipitable with phosphotungstic acid. This value approaches that found in all the other cases after autolysis *in vitro* has proceeded for from one to three days.

The control figures of the five day necrosis, as shown by Experiments 43 and 49, also indicate a high percentage of the total nitrogen of the tissue as monamino-nitrogen. As, however, an unusually large amount of the total nitrogen occurs in non-coagulable form the percentage relation of the monamino acids to the latter is about normal. These lesions were very extensive but of the focal type and the cells at the fifth day were undergoing repair. Hence, although autolytic processes were going on in the tissue at that time, the products were removed as fast as they were formed and no increase in amount occurred in the organ.

The same differences in the velocity and degree of autolysis *in vitro* between the washed and unwashed organs are also very evident and require no discussion since they are due to the same causes. It is interesting to note that the nitrogen occurring as monamino-acids reaches at the end of the first day about 80 per cent. of the non-coagulable nitrogen and then although autolysis may greatly increase, their formation increases only in the same proportion. The exception to this is in case of Dog 48, already discussed, where the percentage was high in the tissue itself and remained at the same level during autolysis *in vitro*. This would seem to point to the fact that as far as monamino-acids are concerned their formation in autolysis *intra vitam* occurs in the same manner and by the same chemical processes as they do in autolysis *in vitro*.

v. Drjewezki found that at the end of seventy-two hours autolysis the monamino-acids took up about sixty per cent. of the total nitrogen of the tissue. This figure is somewhat higher than we obtained at this stage of autolysis but in some instances it was reached on the fifth or eighth day.

Ammonia.—As a result of the work of Loewi,[14] Jacoby,[15] Lang and others, considerable interest has become attached to the power of the surviving liver tissue to produce ammonia, especially in view of the current opinion as to the importance of this product, after it has been split off from the amino-acids, as a step in the formation of urea. This interest has been heightened by the appearance of ammonia in increased absolute and percentage amounts in the urine in certain hepatic disorders, thus apparently bringing these matters into correlation.

We have studied the question in two ways. First, in connection with the nitrogen partition of the autolysis now under discussion, and secondly, after the manner of the discoverer[15] of the ammonia-forming power of the liver. This latter part of the work will be discussed separately.

In the partition tables it is seen that although the ammonia content of the necrotic livers is greater than that of the normal it runs parallel with the increase in the amount of non-coagulable nitrogen. Hence the percentage figures show no regular increase or diminution though variations occur owing to the large limit of error dependent on the small amounts of ammonia formed. A comparison of a forty-eight hour necrotic liver (56), which offers an exception to the above statement, in that it shows a progressive diminution with a normal washed liver (53), is instructive. In 53 the normal percentage of ammonia of the non-coagulable nitrogen runs along at about 4 per cent. throughout the experiment. In 56, however, the control shows a high initial ammonia content (10.2), which on the third day dropped to that of the normal washed liver (53) and remained at that level to the end of the experiment.

[14] Loewi, O., Ueber das Harnstoffbildende Ferment der Leber, *Zeit. f. physiol. Chem.*, 1893, xxv, 511.

[15] Jacoby, M., Ueber die fermentative Eiweissspaltung und Ammoniakbildung in der Leber, *Zeit. f. physiol. Chem.*, 1900, xxx, 149.

We explain this variation in the ammonia formation by the assumption that this tissue contained *intra vitam,* as the result of the necrosis, proportionately larger amounts of ammonia liberating compounds than the normal. As, however, the autolysis proceeded less of these products were formed in relation to the non-coagulable nitrogen than was the case in the autolysis of the normal. Hence the percentage figure dropped. Or if we allow for the initial difference we find that the percentage increase is the same in both cases.

The great increase in the amount of non-coagulable nitrogen which occurred between the first and third day (18.5 to 65.1 per cent.) in Dog 56 could not have included the formation of ammonia compounds, since the increase of these latter bodies in relation to the total nitrogen was so slight that there occurred an actual percentage decrease in relation to the non-coagulable nitrogen.

All this would seem to indicate that the production of ammonia which occurs in the autolysis of the liver *in vitro* is the result of a decomposition of coagulable nitrogen in the cell. That is deamidization of the amino-acids and the splitting of urea does not take place to any greater extent in the necrotic tissue undergoing autolysis than it does in the normal.

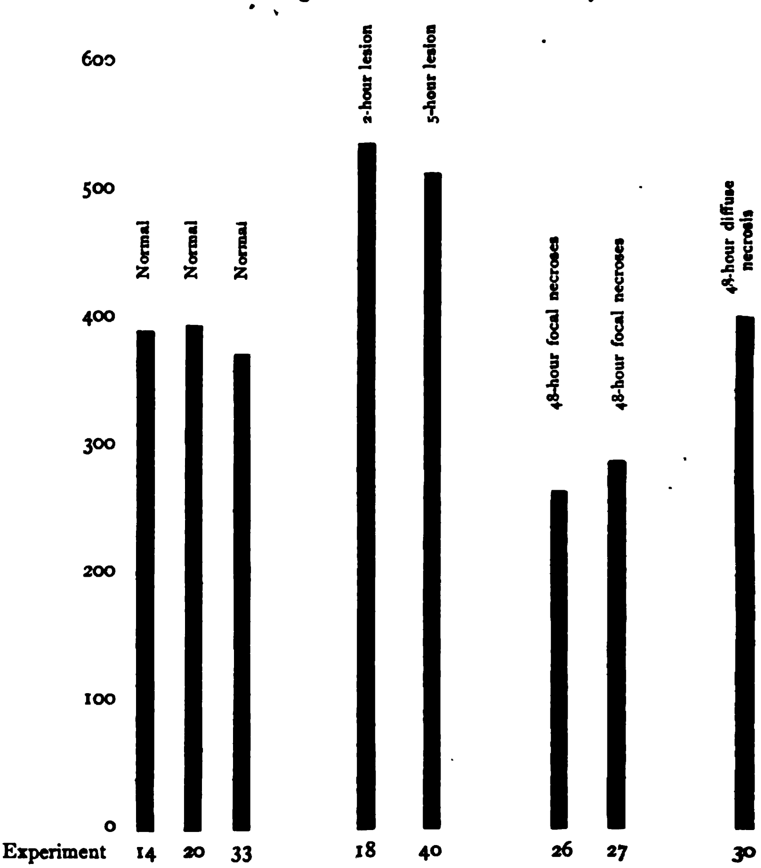
Separate Ammonia Determinations.—These experiments were some of the earlier ones performed and were carried out after the fashion of the investigations reported by Jacoby. A weighed amount, 200 grams, of the finely divided fresh liver tissue was made up to 600 cubic centimeters with distilled water and the well-mixed fluid divided into twelve equal parts. Four of these samples were sterilized immediately for controls. Two were analyzed at once as initial controls, while two, for the purpose of final controls, were placed with the remainder of the portions in the thermostat. At varying periods, duplicate samples were removed for analysis. The ammonia was determined according to a modification of Shaffer's method for the urine. The accompanying diagram indicates in a schematic way the results of the experiments.

It is somewhat difficult to make comparison of these results with those just reported in the partition series since we have no determination of the amount of non-coagulable nitrogen at the various

TABLE II.

Ammonia.

Percentage increase at the end of ten days.



periods; hence the figures represent only the percentage increase of ammonia nitrogen based on its amount in the control sample. Although Jacoby gives in his tables only the amount of ammonia nitrogen formed without taking into consideration the question of dry solids and nitrogen of the dry residue, a recalculation of his figures from average results of our normal livers indicates that more ammonia nitrogen was formed in our experiments than in his. On the other hand, however, our figures are not as high as those re-

ported by Soetbeer.[16] In opposition to the partition series, our figures indicate a larger amount of ammonium compounds in the initial control sample of the normal tissue than in those with varying degrees of necrosis and degeneration. This seeming anomaly we are unable to understand or explain. As, however, the percentage increase figures upon which the diagram is calculated are based upon the initial control as zero, the variable and anomalous control factor is excluded.

Considered in this way it will be seen that the three normals showed an increase of ammonia-nitrogen over the control of from 378 to 396 per cent., an agreement which serves well as a basis for comparison of the experiments on necrotic and degenerated tissue. In the case of the focal necroses the increase is less than the normal, amounting only to 265 to 290 per cent. On the other hand, the diffusely necrotic tissue evidenced the same power to produce ammonia-forming compounds as the normal. In the two samples of congestion and thrombosis, the lesion being of two to five hours' duration, more ammonia was produced than in the normal. These results would seem to indicate that during the initial stage of the process when the liver is intensely congested an increase in ammonia output must occur. This, however, is not supported by our metabolism experiments.[17]

Uric Acid.—The investigation concerning uric acid has yielded results of not sufficient interest for presentation in a table. The only point of importance is that a gradual diminution occurs which ceases on the third day. Since in the autolysis of uric acid ammonia is formed, this factor must influence to a slight extent the increase in ammonia observed in the partition experiments.

Arginase.—In the endeavor to explain the results in connection with the hexon bases, reported in the first paper of this series, a few experiments were conducted in the attempt to prepare from normal and necrotic livers an active substance, according to the

[16] Soetbeer, F., Ueber einen Fall von akuten Degeneration des Leberparenchyms, *Arch. f. exper. Path. u. Pharm.*, 1903, l, 294.

[17] See third paper of this series "Nitrogenous Metabolism" in this number of the *Journal.*

method of Kossel and Dakin,[18] which would hydrolyze arginin into ornithin and urea. Preparations were made by both the ammonium sulphate and acetic acid-ether methods outlined by these investigators and solutions of these were added in aliquot parts to an arginin solution of known strength. The determinations were made sometimes upon the phosphotungstic precipitate, sometimes upon the filtrate from this and once upon both. The aliquots were allowed to autolyze for one, three and five days and controls were done at the beginning and at the end of the experiment.

TABLE III.

Arginase.

Experiment.	Lesion.	Method of Preparation.	Estimation on Phosphotungstate.	c.c. N/10 Acid.			
				Control.	1 day.	3 days.	5 days.
15	Normal.	¾ Saturation $(NH_4)_2SO_4$ precipitate.	filtrate.	1.6	2.65	2.75	2.65
15	Normal.	ditto.	filtrate.	4.55	5.95	6.25	6.80
15	Normal.	ditto.	precipitate.	5.75	4.45	4.25	3.65
19	Normal.	ditto.	precipitate.	4.50	3.40	5.00	5.65
42	Normal.	Extraction acetic acid.	filtrate.	5.20	4.45	4.15	4.30
18	2 hours.	Complete saturation $(NH_4)_2SO_4$ precipitate.	precipitate.	11.45	12.10	12.40	9.90
40	5 hours.	Extraction acetic acid.	filtrate.	5.40	4.65	5.30	4.40
16	Focal necroses.	Complete saturation $(NH_4)_2SO_4$ precipitate.	precipitate.	6.75	6.70	6.55	5.70
28	Diffuse necrosis.	ditto.	precipitate.	6.10	6.90	6.95	6.10
28	Diffuse necrosis.	Extraction dilute HCl.	precipitate.	8.05	9.80	8.45	8.60

Our results in regard to the normal liver agree with those of Kossel and Dakin. The preparations from necrotic livers gave negative or doubtful results, thus affording valuable confirmatory evidence of the position which we assumed as a result of our work upon the hexon bases, namely, that in extreme diffuse necrosis where large areas remote from the circulation are undergoing necrosis

[18] Kossel, A. and Dakin, H. D., Ueber die Arginase, *Zeit. f. physiol. Chem.*, 1904, xli, 321. Weitere Untersuchungen ueber fermentative Harnstoffbildung, *ibid.*, 1904, xlii, 181.

there occurs a marked increase in hexon base content of the tissue. In such areas, evidently, the arginin is not split up to any noticeable extent. The experiments of Wakeman[19] do not show the presence of arginin to the marked extent which the author claims, as we have explained in our discussion of the hexon bases.[20]

The table shows that of the ten preparations made from seven different livers, three normal, two necrotic and two with thrombosis, only one, the normal (15), showed any activity. In the others either no results were obtained or were so irregular that the experiments may be considered as negative.

In this one experiment with normal liver in which the precipitate obtained by complete saturation of the three-quarter saturated filtrate with ammonium sulphate was employed, the nitrogen of the filtrate from the phosphotungstic precipitate showed a gradual increase presumably due to the autolysis of the arginin added. These results would indicate that an active arginase can be obtained only from normal tissue. This is in complete accord with the results reported in the paper on the hexon bases in which it is shown that during the autolysis of necrotic liver tissue an increase in the hexon base content of the cell occurs.

Leucin and Tyrosin.—In view of the well-recognized presence at times of monamino-acids in the urine of individuals suffering with hepatic disorders, particularly acute yellow atrophy, and of the varying results reported by the different observers in regard to the question of the presence of these compounds in the liver tissue, it seemed advisable to examine the urine and the liver of the animals under observation as to the presence of leucin and tyrosin.

It would appear to be unnecessary for us to enter into a discussion of the older literature concerning the variation in the results as to the presence or absence of leucin and tyrosin in the urine under many pathological conditions. This subject has been well discussed by Ewing and Wolf,[21] who conclude that the differences observed

[19] Wakeman, A. J., On the Hexon Bases of Liver Tissue under Normal and Certain Pathological Conditions, *Jour. of Exper. Med.*, 1905, vii, 292.

[20] See first paper of this series, "Hexon Bases" in this number of the *Journal.*

[21] Ewing, J. and Wolf, C. G. L., The Clinical Significance of the Urinary Nitrogen, *Amer. Jour. of the Med. Sciences,* 1906, cxxxi, 751.

are most probably due to faulty methods of technique and of confirmation. Of the recent work, to which this criticism cannot properly be applied is that of Taylor,[22] who found these monamino-acids present in the liver of acute yellow atrophy as well as in that of probable chloroform poisoning. In both instances, supposedly, necrosis of varying degree had taken place. Wells[23] in a preliminary communication confirms these results for acute yellow atrophy. On the other hand, however, in other conditions to which he gives the general term of " degeneration," Taylor failed to find these substances. Again leucin and tyrosin usually appear in the urine of persons or animals poisoned with phosphorus and this fact has been associated with the occurrence of the well-known hepatic changes, chiefly fatty infiltration, which are known to occur in this condition.

The recent method devised by Fischer and Bergell, in which β-naphthalin sulphochloride is employed, and Abderhalden and Barker's modification of Fischer's esterification method are so time-consuming that we decided that for the purpose in view, the simpler methods were of sufficient accuracy to warrant their use. Ewing and Wolf in the paper mentioned above criticize severely the lead acetate method, originally employed by Frerichs and Stădeler. They claim that the microscopic demonstration of leucin and tyrosin by this procedure is unreliable and the crystals supposed to be leucin may be in reality urates or urea. We have used a modification of the lead acetate method in which after the removal of the excess of lead by means of hydrogen sulphide the filtrate is evaporated to dryness and the residue extracted with several portions of absolute alcohol to remove the urea, after which it is treated with repeated portions of ammoniacal absolute alcohol. The united extracts are allowed to evaporate almost to dryness, when characteristic crystals appear, if leucin or tyrosin is present in the original material. When sufficient quantities were present these microscopic findings were controlled by the usual chemical tests. We feel reas-

[22] Taylor, A. E., Ueber das Vorkommen von Spaltungsprodukten der Eiweisskorper in der degenerirten Leber, *Zeit. f. physiol. Chem.*, 1902, xxxiv, 580. On the Occurrence of Amino-acids in Degenerated Tissue, *Univ. of California Publications*, 1904, i (Path.), 43.

[23] Wells, H. G., The Composition of the Liver in Acute Yellow Atrophy. Communication read at the first meeting of the Amer. Soc. of Biol. Chemists, Washington, May 8, 1907.

onably sure that the substances upon which we have based the following results were leucin and tyrosin.

TABLE IV.

Leucin and Tyrosin in the Urine.

Experiment.	Lesion.	Leucin.	Tyrosin.	Urine of
2	No necroses	—	+	4th day
34	No necroses	+++	+	1st and 2d day
1	Focal necroses	+	+	1st day
5	Focal necroses	—	+	1st day
23	Focal necroses	++	+	1st and 2d day
32	Diffuse necrosis	—	+	1st day
48	Diffuse necrosis	—	++	1st and 2d day
51	Diffuse necrosis	—	++	2d and 3d day

TABLE V.

Leucin, Tyrosin and Proteoses in the Liver.

Experiment.	Lesion.	Leucin.	Tyrosin.	Proteoses.	Age of Lesion.
16	Focal necroses	—	+	+	48 hour
32	Diffuse necrosis	—	+	+	26 hour
48	Diffuse necrosis	—	++	+	48 hour
51	Diffuse necrosis	—	++	+	48 hour

The table giving the results of the examinations of the urine shows that there is no regularity in the occurrence of these compounds. The type of the lesion has apparently no relation to the amount eliminated and the results presented justify the general concensus of current opinion that the appearance of these compounds is not to be regarded as pathognomonic of any one condition such as acute yellow atrophy or phosphorus or chloroform poisoning. In addition to the positive results presented in the table the urine of nine other animals was examined with negative results. In five of these the liver showed necroses, in four none.

The results of the examination of the liver substance point to the occurrence of tyrosin in larger amounts when the lesion was most pronounced; thus in each of three livers with diffuse necrosis it was present, but in only one of the five examples of focal necroses did it occur. A normal liver and also one with degeneration but no necroses were likewise negative. In no condition did we find leucin. In four livers with extensive necrosis proteoses were found in considerable quantities while a normal liver yielded none. All of

this is in agreement with the variable results of Taylor mentioned elsewhere.

It is evident, therefore, that leucin and tyrosin may be formed during the autolysis of the hepatic tissue, but their appearance in the urine or detection in the liver is dependent upon the condition of the hepatic cells not involved in the lesion. If these cells can take care of large quantities of monamino-acids carried to them normally by the portal vein we see no reason why, if they are present in sufficient numbers and properly functionating, that they should not react in the same way with the same acids formed during the autolysis. The appearance of these monamino-acids under any condition then would depend upon the quantitative relation of the necrosis to the actively functionating cells which are unaffected by the lesion.

Of considerable interest in connection with the finding by Salkowski[24] in the urine of various pathological conditions, more particularly a case of yellow atrophy, of an increased amount of nitrogen precipitable by alcohol, is the fact that although in the normal liver the residue remaining after the extraction with absolute alcohol and ammoniacal alcohol is small in amount, in the case of the necrotic tissues this amount is markedly increased. We have examined the residue as to its character and find that it consists mainly of proteoses. The removal of these compounds by way of the blood-stream would cause an increase in the urine of undetermined nitrogen usually ascribed to amino-acids. This occurrence explains those conditions characterized by a high rest-nitrogen without the presence of monamino-acids.

SUMMARY.

1. The presence of blood serum has a decided inhibitory effect on autolysis. Thus in the normal unwashed organs the non-coagulable nitrogen increase was 100 to 300 per cent., while in the washed it amounted to 450 per cent. The washed necrotic livers showed an increase of from 600 to 850 per cent., while that of the unwashed necrotic was only slightly above the normal unwashed.

2. While the initial amount of non-coagulable nitrogen varies it is greater in those livers showing the more extensive forms of

[24] Salkowski, E., Zur Kenntnis der Alkoholunlöslichen bzw. kolloidalen Stickstoffsubstanzen im Harn, *Berl. klin. Woch.*, 1905, xlii, 1581, 1618.

necrosis. The final amount of autolysis is also greatest in livers of this type. As regards the rate of autolysis fifty per cent. of the total occurs in the first day in the normal and in all types of lesions both washed and unwashed. The maximum is usually reached on the third day in the unwashed, while in the washed there is a continued increase to the eighth day. At this time in the necrotic livers about two to three times as much of the total nitrogen is in the form of non-coagulable nitrogen as in the normal.

3. In the necrotic tissue the initial controls show the content of monamino-acids, with one exception, to be practically doubled. In the washed necrotic the final amount is seventy per cent. of the total nitrogen against forty-six to fifty-seven per cent. in the washed normal. In all cases the monamino-acid nitrogen runs parallel to the nitrogen in non-coagulable form, but in relation to the total nitrogen it shows a greater increase in the washed than in the unwashed organs.

4. The ammonia production in the necrotic livers as shown by the partition experiments is greater than that in the normal and this increase corresponds to that of the non-coagulable nitrogen. In the experiments concerning the absolute production of ammonia in the presence of serum a greater amount was produced in the two and five hours' lesions than in the normal livers. On the other hand, the forty-eight hour diffuse necrosis equaled the normal and the focal fell below.

5. Arginase was obtained from normal but could not be isolated from necrotic livers.

6. No constant relation could be demonstrated between the anatomical lesion in the liver and the presence of leucin and tyrosin in the urine. Leucin was found occasionally in the urine, but none in the liver. On the other hand, tyrosin was constantly present in livers with diffuse but rarely in those with focal necrosis. In the instances of diffuse necrosis in which the liver and urine of the same animal were examined tyrosin was found in both.

7. The presence of large amounts of proteoses in the necrotic liver indicates that the elimination of these substances (colloidal nitrogen of Salkowski) under such circumstances may account for a part of the total nitrogen of the urine usually attributed to the monamino-acids.

EXPERIMENTAL LIVER NECROSIS; III. NITROGENOUS METABOLISM.[1]

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We present here the results of experiments carried out upon dogs in which the general state of the nitrogenous metabolism of the animals was studied before and after the injection of hæmotoxic immune sera. As has been stated elsewhere[2] the important organic lesion produced by such sera is a hepatic necrosis either focal or diffuse, according to the amount and strength of the serum employed. Such an experimental lesion seemed peculiarly adapted to the study of the function of the liver in general metabolism and of certain peculiar metabolic derangements which have an analogy to those noted in eclampsia, chloroform poisoning, vomiting of pregnancy and acute yellow atrophy. It must be clearly borne in mind, however, that this experimental method of producing hepatic necrosis allows of the infliction upon the liver of a single injury, in point of time, which if not followed by death is rapidly repaired and which is almost without other disturbing factors. Hence metabolic variations may be transient and last but one or two days, and for this reason the resulting disturbance is not comparable to that produced by a continually acting cause with progressive lesion.

The experiments were carried out upon dogs kept in the usual

[1] Conducted under grants from the Rockefeller Institute for Medical Research. Read before the American Association of Pathologists and Bacteriologists, Washington, May 7, 1907. Received for publication July 2, 1907.

[2] See first paper of this series, "The Hexon Bases," in this number of the *Journal.*

well-ventilated metabolism cages and fed upon a purin-free diet of casein, cracker dust and lard. The amounts of the three dietary constituents varied for the different animals. They were calorifically sufficient and so regulated that the animal finally came into nitrogen equilibrium. The dogs were catheterized at the end of each twenty-four hour period and the urine thus obtained, added to that voided naturally. This was at all times carefully preserved from changes of any kind until the analytic work was completed. To the total twenty-four elimination was added distilled water to make the volume up to 800 cubic centimeters. When hæmoglobinuria or albuminuria caused the appearance of proteid in the urine this was removed by heat and acetic acid, the coagulum being thoroughly boiled out with water and the washings added to the urine.

Upon these twenty-four hour samples the following determinations were made: total nitrogen by the Kjeldahl-Gunning method; ammonia by the Shaffer method; urea by the Mörner-Sjoquist method; uric acid by the Hopkins-Folin method and creatinin by Folin's colorimetric adaptation of Weyl's qualitative test. The difference between the sum of these various factors and the total nitrogen is given as rest or undetermined nitrogen. Kynurenic acid was also looked for qualitatively in order to obtain evidence as to variations in the output of this substance.

The general procedure in these experiments was to place the animal upon nitrogen equilibrium and to conduct control determinations for a period of three days, after which the animal was injected with either normal or toxic sera and the experiment allowed to continue until death ensued or the metabolism had regained its normal level as shown by the control period. It is obvious that in experiments of this character it becomes extremely difficult, if not impossible, owing to variance in the toxicity of the sera and the susceptibility of the animals, to regulate the severity of the lesion, especially as the degree of the latter can be determined only by postmortem examination. This fact prohibits the production in any two experiments, no matter how carefully planned, of absolutely comparable pathological conditions. This difficulty is brought out by the fact that although each new lot of serum was tested for lethal action on dogs of approximately the same weight and conditions as

those prepared for the experiment, only six out of twelve of the latter survived the first twenty-four period. These six, with a control dog receiving normal serum, constitute the experiments on which this communication is based.

These may be divided into four groups: one injected with normal serum in which no histological change took place; one with a weak toxic serum which caused no necrosis, but an extensive granular and vacuolar degeneration; four with toxic sera causing more or less extensive focal necroses and one with toxic serum producing diffuse necrosis.

TABLE I.

(*Dog 18, normal serum.*)

Date.	Total Nitrogen.	Urea.*	Ammonia.	Uric Acid.	Creatinin.	Undetermined Nitrogen.	Notes.
Nov. 10	6.51	4.99 / 76.8	0.556 / 8.5	0.018 / 0.28	0.677 / 10.4	0.269 / 4.0	
11	6.09	4.81 / 78.9	0.492 / 8.1	0.013 / 0.21	0.633 / 10.4	0.142 / 2.4	
12	6.52	5.25 / 80.5	0.493 / 7.6	0.020 / 0.30	0.607 / 9.3	0.150 / 2.3	Injected 12 M. Dose 1: 738. Vomited.
13	6.60	5.06 / 76.6	0.510 / 7.7	0.017 / 0.26	0.576 / 8.7	0.437 / 6.7	
14	6.06	4.72 / 77.8	0.482 / 8.0	0.021 / 0.34	0.539 / 8.9	0.298 / 5.0	

* Figures in upper left-hand corner represent grams nitrogen; those in lower right-hand corner, percentage of total nitrogen.

Table I shows the result of the injection of normal serum. With this may be compared also Tables VI and VII, in which it is seen that the toxic serum was preceded by an injection of normal serum. In two of these the dose[8] of the normal serum was greater than that of any injection of toxic serum. It will be seen that it was practically without effect although in Dog 48 (Table VI) a slight increase in the output of total nitrogen was evident. A consideration of the nitrogen partition indicates, however, that this increase is mainly at the expense of the rest-nitrogen and is to be explained by traces of foreign proteid of the rabbit's serum injected, too small to be removed by the ordinary methods. We possess, there-

[8] The figures representing dosage, for example 1:600, indicate that the dose was in the proportion of one cubic centimeter of serum to 600 grams of body weight.

fore, a series of controls upon which to base our conclusions concerning the effect of the toxic sera.

Table II gives the figures obtained as the result of injecting a weak serum,[4] which caused extensive hepatic degeneration of the granular and vacuolar type but no necrosis.

TABLE II.

(*Dog 12, Degeneration, No Necrosis.*)

Date.	Total Nitrogen.	Urea.*	Ammonia.	Uric Acid.	Creatinin.	Undetermined Nitrogen.	Notes.
Oct. 15	4.33	3.67 84.5	0.157 3.6	0.006 0.14	0.302 7.0	0.195 4.76	
16	3.77	3.24 85.7	0.131 3.5	0.005 0.13	0.285 7.6	0.109 3.07	
17	3.76	3.29 87.3	0.120 3.1	0.004 0.13	0.284 7.6	0.062 1.87	
18	3.16	2.72 87.1	0.104 3.3	0.004 0.13	0.241 7.6	0.060 1.87	
19	4.23	3.56 84.1	0.090 2.1	0.004 0.09	0.315 75.	0.261 6.21	Injected 4.30 P.M; weak toxic serum; dose 1:715.
20	3.37	2.66 78.9	0.165 4.9	0.003 0.09	0.288 8.5	0.254 7.66	
21	3.19	2.69 84.3	0.161 5.1	0.006 0.19	0.268 8.4	0.065 2.01	
22	3.02	2.60 86.1	0.122 4.0	0.002 0.06	0 260 8.7	0.036 1.14	Killed.

* Figures in upper left-hand corner represent grams nitrogen; those in lower right-hand corner, percentage of total nitrogen.

It is seen that a slight but transient rise in the total nitrogen occurred. A small part of this increase is attributable to the foreign proteid injected, with a corresponding rise in the undetermined nitrogen, both in absolute and percentage amounts. The absolute amount of urea nitrogen increased during the first twenty-four hours after injection but not in sufficient quantities to keep pace with the increase in total nitrogen, hence a percentage decrease occurred. The following day both the percentage and absolute amounts diminished markedly to be followed on the succeeding days by return to the normal percentage of urea output. The absolute quantity remained low since the total nitrogen did not return to nor-

[4] The serum of a rabbit which was not bled until six weeks after immunization against dog's blood. Sera obtained so long after injection frequently show diminished hæmagglutinative and hæmolytic power.

mal. The ammonia output during the twenty-four hours succeeding the injection suffered a decided diminution both in percentage and absolute figures. This decrease was exceedingly transient, since on the succeeding days the reverse occurred and both the absolute and percentage figures went much above the normal. The uric acid and creatinin showed no change but the rest-nitrogen increased considerably on the first and second days following the injection.

The reaction[6] for kynurenic acid was positive on the day subsequent to the injection and passed off gradually. This would indicate an increase in proteid destruction.[7]

Of most importance is the diminution in the percentage of the total nitrogen eliminated as urea associated with a somewhat corre-

TABLE III.

(*Dog 25, Focal Necroses.*)

Date.	Total Nitrogen.	Urea.*		Ammonia.		Uric Acid.[5]	Creatinin.		Undetermined Nitrogen.		Notes.
Dec. 2	4.77	3.55	76.5	0.443	9.1		0.405	8.4	0.372	6.0	
3	5.42	4.39	81.0	0.507	9.4		0.362	6.7	0.161	3.3	
4	5.24	4.11	78.4	0.440	8.4		0.298	5.7	0.392	7.5	
5	4.97	3.86	77.6	0.421	8.5		0.335	6.7	0.354	7.2	
6	6.02	4.68	77.4	0.496	8.2		0.362	6.0	0.482	8.4	Injection 2:30 P.M. toxic serum dose 1 : 1000. Vomited.
7	5.85	4.77	81.5	0.443	7.6		0.351	6.0	0.286	4.9	Hburia.
8	5.92	4.95	83.6	0.313	5.3		0.323	5.3	0.334	5.8	Hburia.
9	9.33	7.66	82.1	0.587	6.3		0.430	4.6	0.653	7.0	Hburia.
10	6.46	5.40	83.5	0.376	5.8		0.360	5.6	0.324	5.1	
11	8.15	7.16	87.8	0.404	4.9		0.368	4.5	0.218	2.8	
12	8.22	7.07	86.0	0.461	5.6		0.366	4.5	0.323	3.9	No food taken; killed.

* Figures in upper left-hand corner represent grams nitrogen; those in lower right-hand corner, percentage of total nitrogen.

[5] Amounts so small that they were not calculated.

[6] Treatment of the urine with bromine water.

[7] Mendel, L. B. and Jackson, H. C., On the Excretion of Kynurenic Acid. *Amer. Jour. of Physiol.*, 1898, ii, 1.

TABLE IV.

(Dog 43, Focal Necroses.)

Date.	Total Nitrogen.	Urea.*		Ammonia.		Uric Acid.		Creatinin.		Undetermined Nitrogen.		Notes.
Feb. 2	7.74	6.31	81.5	0.444	5.7	0.018	0.23	0.417	5.4	0.551	7.2	
3												Fæces mixed with urine.
4	6.95	5.50	79.1	0.406	5.8	0.013	0.17	0.338	4.9	0.693	10.0	
5	6.45	5.37	83.2	0.335	5.2	0.015	0.23	0.280	4.3	0.450	7.1	Injected 10:30 A.M. toxic serum dose 1 : 1738. Vomited.
6	9.91	8.48	85.5	0.478	4.8	0.073	0.73	0.343	3.5	0.836	8.5	Injected 3 P.M. toxic serum; dose 1 : 1200.
7	10.56	8.88	84.1	0.578	5.5	0.045	0.42	0.405	3.8	0.652	6.2	
8	7.65	6.31	82.5	0.479	6.2	0.009	0.12	0.386	5.0	0.466	6.2	
9												Urine lost.
10	4.85	3.84	79.2	0.288	5.9	0.007	0.14	0.341	7.2	0.374	7.6	Killed.

* Figures in upper left-hand corner represent grams nitrogen; those in lower right-hand corner, percentage of total nitrogen.

sponding increase in the percentage output of ammonia and rest-nitrogen. This is the urinary picture which recently has been described as associated with the hepatic disorder supposed to underly the symptoms of chloroform poisoning, toxæmia of pregnancy and like conditions and which will be discussed more in detail after our results have been completely given.

Tables III, IV, V, VI and VII present the results of the experiments in which a true necrosis, either focal or diffuse, was obtained.

In these experiments the injection of the toxic sera[8] was always quickly followed by a more or less marked increase in the elimination of total nitrogen which *persisted* for several days. During the first or second twenty-four hour period, after injection, occurred a slight increase in the percentage of urea nitrogen (three to five per cent.) which was followed on the succeeding days by a drop to normal, and in one experiment below normal. The ammonia nitro-

[8] In three of these experiments the first injection was followed by a second after a varying interval. This fact renders the figures after the time of the second injection less comparable.

gen percentage of the total nitrogen diminished gradually after the injection and reached its lowest point about the second or third day, after which, in some cases, as in Dogs 49 and 43, it returned to normal; in others, as in Dogs 25, 45 and 48, it remained low. The more advanced the repair at the time of death, the nearer the percentage of ammonia nitrogen had returned to the normal.

The uric acid nitrogen suffered a marked, though transient, increase.[9] In the three experiments where successive injections were given, the second injection in each instance caused an increase of uric acid on the following day after which it returned to normal.

The absolute creatinin nitrogen output was noticeably augmented after injection; this increase, however, was not quite in the same proportion as the total nitrogen, hence the creatinin nitrogen per cent. of the total tended at times to show a slight diminution.

TABLE V.

(Dog 45, Focal Necroses.)

Date.	Total Nitrogen.	Urea.*	Ammonia.	Uric Acid.	Creatinin.	Undetermined Nitrogen.	Notes.
Feb. 3	6.70	5.50 82.1	0.342 5.1	0.014 0.21	0.274 4.1	0.570 7.5	
4	6.57	5.37 81.7	0.491 7.5	0.013 0.20	0.296 4.5	0.400 6.1	
5	6.43	5.05 78.5	0.452 7.0	0.012 0.18	0.274 4.1	0.642 10.2	
6	6.52	5.33 81.7	0.455 7.0	0.013 0.20	0.265 4.1	0.457 7.0	Injected 10 A. M. toxic serum; dose 1:1500
7	8.99	7.51 83.5	0.560 6.2	0.105 1.17	0.359 4.0	0.456 5.1	
8	7.97	6.25 78.4	0.524 6.6	0.010 0.13	0.300 3.9	0.886 11.0	Injected 10 A. M. toxic serum; dose 1:1000
9	5.30	4.01 75.6	0.441 8.3	0.055 1.04	0.298 5.7	0.928 9.4	
10	10.30	8.20 79.6	0.646 6.3	0.030 0.28	0.349 3.4	1.075 10.4	
11	8.37	6.71 80.2	0.598 7.1	0.021 0.25	0.313 3.7	0.728 8.8	
12	7.45	5.99 80.0	0.447 6.0	0.009 0.12	0.326 4.5	0.678 9.4	Killed

* Figures in upper left-hand corner represent grams nitrogen; those in lower right-hand corner, percentage of total nitrogen.

9 For a detailed discussion of this subject see fourth paper of this series, "Nuclein Metabolism," in this number of the *Journal.*

TABLE VI.

(Dog 48, Diffuse Necrosis.)

Date.	Total Nitrogen.	Urea.*	Ammonia.	Uric Acid.	Creatinin.	Undetermined Nitrogen.	Notes.
Feb. 19	6.80	5.90 86.7	0.374 5.5	0.018 0.26	0.373 5.5	0.135 2.0	
20	6.55	5.53 84.4	0.360 5.5	0.018 0.27	0.364 5.6	0.278 4.2	
21	6.38	5.41 84.8	0.358 5.6	0.024 0.37	0.326 5.2	0.228 4.0	Injected 5 P. M. normal serum; dose 1:600.
22	7.00	5.76 82.3	0.406 5.8	0.024 0.34	0.338 4.8	0.472 6.8	
23	6.53	5.50 84.2	0.376 5.8	0.021 0.32	0.326 5.0	0.307 4.7	
24	6.10	5.00 82.0	0.400 6.6	0.018 0.30	0.314 5.1	0.368 6.0	Injected 10 A. M. toxic serum; dose 1:1155.
25				0.036			Vomitus mixed with urine.
26	8.78	7.58 86.3	0.365 4.1	0.018 0.20	0.345 4.0	0.472 5.4	
27	9.11	6.80 (?) 76.4	0.551 6.0	0.018 0.20	0.349 3.8	1.392(?) 13.6	Injected 10 A. M. toxic serum; dose 1:600. Vomited.
28	11.12	9.24 83.1	0.418 3.8	0.130 1.17			Hburia. Vomiting; refused food.
Mar. 1				0.083			Died.

* Figures in upper left-hand corner represent grams nitrogen; those in lower right-hand corner, percentage of total nitrogen.

The undetermined or rest-nitrogen which in a general way may be said to indicate the output of amino-acids, polypeptids or proteose-like bodies[10] also underwent a decided increase after injection.

An increase in kynurenic acid elimination after injection was noticed at times, but this was slight at the best and in no way corresponds to the increase which occurs after the administration of phosphorus, phlorhizin and large quantities of meat. In these latter instances Mendel and Jackson[11] showed that the increased kynurenic acid output was associated with augmented endogenous

[10] Salkowski, E., Zur Kenntnis der Alkohlolunlöslichen bzw. colloidalen Stickstoffsubstanzen im Harn, *Berl. klin. Woch.*, 1905, xlii, 1581, 1618.

[11] Mendel, L. B. and Jackson, H. C., On the Excretion of Kynurenic Acid, *Amer. Jour. of Physiol.*, 1898, ii, 1.

or exogenous destruction of proteid material containing the tyrosin nucleus.

As has already been stated, we believe that the effect of the blood changes produced by the serum is directed almost entirely upon the liver and represents a single attack upon this organ. Two coincident conditions, which however do not affect the results in any manner, require perhaps a brief notice. In the first place, vomiting usually occurs and persists for a short time, five to ten minutes, after the injection. The feeding and injection were so arranged that nothing was lost in this manner and as the vomiting also occurred when normal serum was used, with no apparent effect on the metabolism, we believe this factor may be disregarded. It has occasionally, however, caused the loss, on account of admixture of vomitus, of a day's urine.

TABLE VII.

(*Dog 49, Focal Necroses.*)

Date.	Total Nitrogen.	Urea.*	Ammonia.	Uric Acid.	Creatinin.	Undetermined Nitrogen.	Notes.
Feb. 22	5.24	4.33 82.6	0.366 7.0	0.021 0.40	0.215 4.1	0.308 5.9	
23	5.48	lost	0.320 5.9	0.018 0.33	0.218 4.0		
24	5.46	4.41 80.8	0.345 6.3	0.021 0.38	0.208 3.8	0.476 8.7	Injected 10 A. M. normal serum; dose 1:600
25				0.018			Vomitus mixed with urine
26	5.25	4.34 82.7	0.297 5.7	0.021 0.40	0.194 3.7	0.398 7.5	Trace albumin
27	5.63	4.77 84.8	0.320 5.7	0.022 0.39	0.204 3.6	0.314 5.5	Injected 10 A. M. toxic serum; dose 1:628. Vomited
28	7.07	6.07 85.6	0.245 3.5	0.014 0.20	0.189 2.7	0.452 8.0	Hburia
Mar. 1	9.14	7.56 82.7	0.270 2.8	0.029 0.32	0.225 2.5	1.056 11.7	Hburia
2	8.45	6.98 82.6	0.428 5.1	0.017 0.20	0.221 2.6	0.804 9.5	Hburia
3	6.97	6.04 86.6	0.370 5.3	0.022 0.31	0.214 3.1	0.324 4.7	Hburia
4	5.70	4.92 86.3	0.366 6.4	0.020 0.35	0.196 3.4	0.198 3.6	Hburia. Killed

* Figures in upper left-hand corner represent grams nitrogen; those in lower right-hand corner, percentage of total nitrogen.

In the second place hæmoglobinuria, sometimes but not always, makes its appearance after twenty-four hours. This condition, however, could not have produced the changes in the general metabolism which we have described since in two of the experiments (43 and 45), where no hæmoglobin or bile appeared in the urine, the results were the same as in those showing a well-marked hæmoglobinuria. This agrees with the observations of Samuely[12] who found in experimental anæmia, produced by means of pyrodin, that the appearance of hæmoglobin or bile in the urine stood in no direct relationship to the changes which took place during the anæmia. On the other hand, Andrea[13] in a series of experiments, in which various hæmolytic substances (phenylhydrazin, pyrogallol, p-phenylendiamin, glycerin) were administered to rabbits, has found an increase of urea after the initial injection, but a decrease of one third after subsequent injections. The increase he explains by destruction of hæmoglobin and the decrease as due to impaired hepatic function.

Another possibility, however, must also be considered. This is that the temporary anæmia which is simultaneously produced as the result of the primary action of the serum on the red cells may originate changes in oxidization capable of accounting for some of the results. If the anæmia were general in character and of the type which occasions a greatly diminished oxidative power throughout the body, such as is noticed after carbon monoxide poisoning, then we should expect to find, among other disturbances, the elimination of incompletely oxidized products of catabolism, such as lactic acid. We have searched for the appearance of this substance in the urines of five of the animals showing necrosis, but have failed to find it. This, with other facts, appear to justify the exclusion of the factor of diminished oxidation.

In this connection it is of considerable interest to note also that in the type of anæmia produced by Samuely the power of the body to oxidize aromatic compounds, such as phenylalanin and cystein,

[12] Samuely, F., Stoffwechseluntersuchungen bei experimenteller Anämie, *Deut. Arch. f. klin. Med.*, 1907, lxxxix, 220.

[13] Andrea, P., Influenca della sostanze emolitiche sulle funzioni ureogenetica ed antitossica del fegato, *Arch. Int. de Pharmacodyn. et de Therapie*, 1905, xiv, 389.

was somewhat decreased, but that the metabolism in regard to the fatty amino-acids was absolutely unchanged.

Upon the whole, therefore, it seems justifiable to designate, as the main causative factor in the production of the results obtained, the necrotic lèsions more or less diffusely distributed throughout the liver.

Several attempts have been made to study the influence of hepatic necrosis upon the metabolism. Jacoby[14] was the first to study the effect of tying off the vessels supplying certain lobes of the liver. Unfortunately the animals did not survive the operation a sufficient length of time to allow observations upon the urine and he was compelled to content himself with demonstrating that products of autolysis were present in the lobes shut off from the circulation. Doyon and Dufourt[15] report that upon tying off the hepatic artery they obtained a diminished formation of urea and increase in ammonia. Their results were somewhat unsatisfactory, however.

From the clinical side of the question quite recently a considerable amount of data which bears upon the question at hand has accumulated. Many investigators have studied the urinary changes occurring in certain metabolic disorders associated with hepatic diseases. Thus Schittenhelm[16] reports that in chronic diseases of the liver the ammonia output in relation to the total nitrogen elimination is increased. Axisa[17] states that in liver abscesses the same change is associated with a marked decrease in the urea percentage. Ingelrans and Dehons[18] corroborate these findings in hepatic insufficiency and claim that cirrhosis gives the same picture as acute yellow

[14] Jacoby, M., Ueber die fermentative Eiweissspaltung und Ammoniakbildung in der Leber, *Zeit. f. physiol. Chem.*, 1900, xxx, 149.

[15] Doyon, M. and Dufourt, L., Contribution à l'étude de la fonction ureopoietique du foie; Effets de la ligature de l'artère hepatique et de alle de la veine porto (*Arch. de physiol. normal et path.*, 1898, S. 5, x, 522), Ref. in *Maly's Jahresbericht f. Thierchemie*, 1898, xxviii, 382.

[16] Schittenhelm, A., Zur Frage der Ammoniakausscheidung im menschlichen Urin, *Deut. Arch. f. klin. Med.*, 1903, lxxvii, 517.

[17] Axisa, E., Ueber Harnstoff und Ammoniakausscheidung im Harn bei Leberabszess, *Zent. f. innere Med.*, 1905, xxvi, 929.

[18] Ingelrans, L., and Dehons, M., La valeur clinique de quelques signes urinaires considérés comme révélateurs de l'insuffisance hepatique, *Arch. de* med. exper. et d'anat. path., 1903, xv, 188.

atrophy. De Rossi,[19] on the other hand, offers evidence that not all diseases in which lesions of the liver are present show this altered relation of urea to ammonia elimination and concludes that the liver is not the only seat of the formation of urea.

In regard to acute yellow atrophy, vomiting of pregnancy, eclampsia, delayed chloroform poisoning and phophorus poisoning, recent investigations seem to show that the hepatic lesions, which are found to be present at autopsy in these conditions, are an important causative factor in the disturbance of metabolism. This disturbance shows itself in the urine by a marked diminution in the output of urea and an increase of the ammonia in relation to the total nitrogen elimination. Williams[20] assumes that the urinary picture of pernicious vomiting of pregnancy with its high percentage ammonia output is sufficiently definite to render it a valuable aid in determining the question of inducing labor. On the other hand, he contends that in the condition known as eclampsia there is a diminution in the total nitrogen and percentage urea with no very pronounced ammonia variation. Stone[21] believes that the vomiting of pregnancy is the result of a toxæmia, the lesions of which are primarily an acute degeneration of the liver amounting sometimes to necrosis and resembling in the fatal cases those of acute yellow atrophy. Zweifel's[22] researches confirm the opinion that the causative factor in eclampsia is a diminished oxidation which shows itself in the production and elimination of considerable quantities of p-lactic acid. The increased ammonia output is the result of the neutralization of the excess of acids produced and the diminished urea is due to the removal of quantities of ammonia which normally would be synthesized into urea.

[19] De Rossi, S., Sul valore semeiologico dell'urea et dell'ammonica nelle lesioni epatiche, *Rifborma Medica*, 1904, xx, 1177.

[20] Williams, J. W., Pernicious Vomiting of Pregnancy, *Surgery, Gynecology and Obstetrics*, 1905, i, 41; *Johns Hopkins Hospital Bul.*, 1906, xvii, 71; *Amer. Jour. Med. Sciences*, 1906, cxxxii, 132.

[21] Stone, W. S., The Toxæmia of Pregnancy, *Amer. Gynecology*, 1903, iii, 518; Some Further Notes on the Toxæmia of Pregnancy, *Med. Record*, 1905, lxviii, 295.

[22] Zweifel, Zur Aufklärung der Eklampsie, *Arch. f. Gyn.*, 1905, lxxvi, 537.

Ewing and Wolf[23] report observations made upon pregnant women from the results of which they conclude that the various conditions of eclampsia, vomiting of pregnancy and yellow atrophy are but different degrees or manifestations of the same disordered process which probably centers itself in the hepatic cells and leads to the deranged elimination of urea and ammonia. A similar disturbance of metabolism associated with necrosis of the liver, has been found by Bevan and Favill[24] in fatal chloroform poisoning.

In view of all this it can readily be seen that the concensus of opinion favors the idea that the changes in the percentage elimination of urea and ammonia which are found to occur in these various conditions are but indications of the same functional lesion which centers itself in the hepatic cell. When necrosis of the liver occurs the cells which ordinarily synthesize ammonia into urea are out of function and the ammonia elimination is increased and the urea correspondingly falls.

With a full appreciation of the necessity of caution in transcribing deductions from the results of animal experiments to the explanation of pathological variations in the human organism, we feel that our results render somewhat doubtful the relationship between the hepatic necrosis of the vomiting of pregnancy, for example, and the urinary finding of a high percentage ammonia output. Wolf[25] has already justly criticized the conclusions of Williams in this regard and emphasizes the well-known fact that equally high percentages of ammonia are to be found when for any reason, as in inanition, the nitrogen or the calorific value of the diet becomes insufficient for the replacement of the wear and tear of the cell.[26] Schittenhelm has shown the influence of diet in this connection by experiments in chronic hepatic diseases where a high ammonia output is present. He noticed that upon increasing the fat of the diet

[23] Ewing, J. and Wolf, C. G. L., The Clinical Significance of the Urinary Nitrogen, II. The Metabolism in the Toxæmia of Pregnancy, *Amer. Jour. of Obstetrics*, 1907, lv, 289.

[24] Bevan, A. D., and Favill, H. B., Acid Intoxication and Late Poisonous Effects of Anæsthetics, *Jour. Amer. Med. Assoc.*, 1905, xlv, 691.

[25] Wolf, C. G. L., The Chemistry of Toxæmias in Pregnancy, *New York Med. Jour.*, 1906, lxxxiii, 813.

[26] Folin, O., Laws Governing the Chemical Composition of Urine, *Amer. Jour. of Physiol.*, 1905, xiii, 66.

a still further increase in the elimination of ammonia occurred and believes that the ammonia offers simply an indication of the lack of normal oxidation or catabolism of the ingested fatty acids. An examination of the results presented by Williams makes it seem very plausible that the diet in his cases is not an unimportant factor in the results. The figures for the total nitrogen indicate that the patients were practically in a state of diminished nutrition even approaching inanition since the amounts fall anywhere between four to eight grams per day, and more important still, the higher the total nitrogen the lower the ammonia and *vice versa* regardless of the severity of the condition. This same criticism can also be applied to the results of Ewing and Wolf. The daily total nitrogen elimination in their experiments is quite as low as that found by Williams, hence the high ammonia can be equally well attributed, in part at least, to similar causes.

The factor of low and insufficient diet was excluded in our experiments since the animals were upon exact equilibrium. We did obtain, however, severe hepatic lesions consisting of localized or diffuse necrotic areas and the ammonia output of our animals never showed more than the merest increase which was exceedingly transient. At this place emphasis must be laid upon the one experiment in which results comparable to, if not as pronounced as those of Williams and of Ewing and Wolf, were obtained. In this instance (see Table II.), however, the histological findings indicated that we were dealing not with a lesion of necrotic character but with an extensive and diffuse degeneration.

On account of the loose use of terms in pathology, this would seem to emphasize that a clear-cut differentiation between degeneration and necrosis[27] must be made histologically if we are to correlate the results of chemical studies and histological findings. The pathological condition "degeneration" does not imply autolysis which occurs only in necrosis. It is evident that one may occur without the other and therefore that the chemistry of the cell depends on its functional activity as determined by its physical state. As a reasonable explanation of why a difference in metabo-

[27] See first paper of this series, "The Hexon Bases," in this number of the *Journal.*

lism must be expected in the conditions of degeneration as opposed to necrosis we would present the following: In a generalized hepatic degeneration the lesion affects the protoplasm of each and every cell of the whole organ without destruction of the nucleus. This degeneration may set up enzymotic disturbances, secondary in character, which are not connected directly with the actual life processes of the cell and which may readily again return to normal when the abnormal conditions of the cell are removed. Such a differentiation between the actual life processes of the cell and those of a secondary functional character finds best expression in the German words "Baustoffwechsel" and "Betriebstoffwechsel." When the disturbance of enzymotic equilibrium occurs, if we grant for the sake of argument the unproven hypothesis that the urea formation is the result of enzymotic relations, there would take place an interference in the production of urea from ammonium compounds without an increase in the output of total nitrogen. A simple rearrangement in the partition factors would evidence itself according to which the ammonia would increase as the urea correspondingly diminished. This is exactly the condition found in our experiment with diffuse degeneration.

On the other hand, in necrosis, the individual cell is destroyed and all its functions cease. Autolysis begins in the same way as it does when death supervenes as the result of the removal of the cell from the body. Under such circumstances there occurs a true protoplasmic decomposition from which the cell can never recuperate. Here the nucleus becomes involved as is shown by the histological picture and in the urine by the occurrence of a marked increase in the elimination of uric acid, purin bases[28] and phosphorus.

In necrosis, moreover, although many individual cells are dead and have ceased to functionate, there always remain, unless the whole organ becomes necrotic, in contradistinction to the condition of degeneration, many normal cells, ready and capable of assuming in a vicarious manner the function of those already dead. This "factor of safety" in the liver is well demonstrated by the partial extirpation experiments of Ponfick, while the power of other organs

[28] For a detailed discussion of this subject see fourth paper of this series. "Nuclein Metabolism," in this number of the *Journal*.

to assume the urea-forming function is shown by the numerous Eck fistula experiments.

In necrosis, therefore, all that is expected as a urinary finding is the appearance of an increase in the total nitrogen output and of the abnormal products of autolysis such as proteoses, polypeptids and amino-acids; and even these latter, in scattered focal necrosis, need not necessarily appear since as very little liver tissue is destroyed the remaining normal cells still possess the power of splitting these substances, formed by cellular digestion, just as they do similar products of intestinal digestion brought to them by the portal[29] vein.

Our results substantiate this theoretical expectation. In the experiments with diffuse necrosis a marked and continued augmentation in the total nitrogen and urea elimination occurred as the result of the removal of the products of autolysis. The diminution in the ammonia output may be ascribed naturally to the increase in the proteid catabolism. Finally the increase in undetermined nitrogen is not definitely to be ascribed to the amino-acids ordinarily considered in this connection since we have not found leucin and tyrosin in the urine in amounts which would compare with those found in acute yellow atrophy.[30] It is to be considered rather as in the "colloidal" form as described by Salkowski and Mancini.[31]

SUMMARY.

1. In focal and diffuse necroses of the liver due to hæmotoxic sera there occurs an increased elimination of total nitrogen with a corresponding augmented output of urea. The ammonia excretion becomes slightly diminished at first, but later rises somewhat above normal. The undetermined nitrogen is markedly increased.

2. In diffuse degeneration with no necrosis on the other hand only a slightly increased output of total nitrogen is evident. A

[29] Freund, E. and Tœpfer, G., Ueber den Abbau des Nahrungseiweisses in der Leber, *Zeit. f. exp. Path.*, 1906, iii, 632.

[30] Riess, L., Phosphorvergiftung und Leberatrophie, *Berl. klin. Woch.*, 1905, xlii (Ewald Festnummer 44 a, 54).

[31] Mancini, S., Studi un nuovo segno per la diagnosi di insufficienza epatica; Contributo allo studio dell'azeta colloidale nelle urine normali e pathologiche, (*Arch. di farmocol. speriment*, 1906) Ref. in *Biochem. Cent.*, 1906, v, 549.

rearrangement of the urea-ammonia proportion occurs in that the ammonia excretion is augmented while the urea elimination is correspondingly diminished. The undetermined nitrogen rises but little.

3. In control experiments with normal serum no effect is produced.

4. These results would appear to indicate that in lesions characterized by uniform degeneration of the liver parenchyma, in contradistinction to necrosis, there occurs no increased nitrogen elimination but merely a disturbance of the urea-forming function of the cell without the appearance in the urine of products of autolysis. On the other hand in necrosis, of even considerable extent, the total-nitrogen is greatly augmented, as is also the rest-nitrogen; while the production of urea, on account of the persistence of normally functioning liver cells, remains relatively unchanged.

This " factor of safety "[32] possessed by the liver is, we think, one of the most important results brought out in this investigation and must be given great weight in any consideration of the chemistry of hepatic disturbances.

[32] Meltzer, S. J., The Factors of Safety in Animal Structure and Animal Economy, *Jour. of Amer. Med. Assoc.*, 1907, xlviii, 655.

EXPERIMENTAL LIVER NECROSIS; IV. NUCLEIN METABOLISM.[1]

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The experiments here detailed were undertaken for the purpose of determining the process or processes intimately concerned in the increased elimination of uric acid which, as has been shown elsewhere,[2] accompanies the augmented output of total nitrogen when hæmotoxic serum is injected. The fact that the principal lesion produced by the toxic serum is in the liver lends peculiar interest to the problem, in view of the important part which this organ is supposed to play in the formation of endogenous uric acid. The direct object of the experiments was to determine whether the increased elimination of uric acid in dogs under these conditions was the result of the breaking down of nuclear material during the necrosis which follows the injection, or whether it signified simply a diminished oxidative power on the part of the hepatic cell by which the uric acid normally oxidized to simple complexes is eliminated unchanged. Or, more concisely, does an actual increase in the production of uric acid from the nucleic acids of the decomposed nuclei of the necrotic cell occur or is there a simple rearrangement of enzymotic equilibrium by which less uric acid is decomposed than normally.

[1] Conducted under grants from the Rockefeller Institute for Medical Research. Received for publication July 2, 1907.

[2] See third paper of this series "Nitrogenous Metabolism" in this number of the *Journal.*

Schittenhelm[3] has isolated two enzymes from the spleen and liver, one of which causes a hydrolytic splitting off of the amino group of guanin and adenin transforming these bodies thereby into xanthin[3] and hypoxanthin respectively; the other an oxydase, xantho-oxidase (Jones), oxidizes the latter compounds into uric acid. Since adenin, guanin, xanthin and hypoxanthin are found in varying amounts in the different nucleic acids which give character to the nucleoproteid of the nucleus we have a fairly definite process by which uric acid results, it may be assumed, from the decomposition of the nucleus by means of autolytic enzymes aided perhaps by a special oxydase.

On the other hand, however, the fact has long been known that various tissues are capable of decomposing uric acid. Wiener,[4] Schittenhelm[5] and Almagia[6] have recently studied the question in detail and describe the presence of uricolytic enzymes in various tissues of several animals. Schittenhelm claims that the calf's kidney is most active in this regard with the liver of the same animal second in power. Pfeiffer[7] was able to prove that ninety to ninety-five per cent. of the uric acid added to human kidneys suffered decomposition. In the horse Almagia found the most active enzyme to be in the liver. These results indicate that the liver among other organs possesses the power of forming uric acid by oxidization and perhaps also by synthesis,[8] while at the same time it is

[3] Schittenhelm, A., Ueber die Harnsäurebildung in Gewebsauszügen, *Zeit. f. physiol. Chem.*, 1904, xlii, 251; Ueber die Harnsäurebildung und die Harnsäurezersetzung in dem Auszügen der Rinderorgane, *ibid.*, 1905, xlv, 121.

[4] Wiener, H., Ueber Zersetzung und Bildung der Harnsäure im Thierkörper, *Arch. f. exper. Path. u. Pharm.*, 1899, xlii, 375.

[5] Schittenhelm, A., Ueber das uricolytische Ferment, *Zeit. f. physiol. Chem.*, 1905, xlv, 161.

[6] Almagia, M., Zur Lehre vom Harnsäurestoffwechsel, Ueber die Zersetzung der Harnsäure durch die Organe des Saugetheirs, *Beit. z. chem. Phys. u. Path.*, 1905, vii, 459.

[7] Pfeiffer, W., Zur Lehre vom Harnsäurestoffwechsel, Ueber die Zersetzung der Harnsäure durch menschliche Nierengewebe, *Beiträge z. chem. Phys. u. Path.*, 1905, vii, 463.

[8] Burian, R., Ueber die oxydative und die vermeintliche synthetische Bildung von Harnsäure im Rinderleber Auszug, *Zeit. f. physiol. Chem.*, 1905, xliii, 497; Die Herkunft der endogenen Harnpurine bei Mensch und Saugethier. *ibid*, 1905, xliii, 532.

capable of decomposing it through the stages of allantoïn and glyoxylic acid.

Austin[9] has raised the point, however, that the alkali in which the uric acid is dissolved is capable of splitting up the latter and that all preparations of uricolytic enzymes contain purin bases which readily become transformed into uric acid. Notwithstanding the latter criticism it seems fairly well founded that the reaction under discussion is a reversible one in which synthetic and analytic processes come into a state of equilibrium in the cell and that alterations in the activity of one or the other set of enzymes increase or diminish the elimination of uric acid without the occurrence, necessarily, of an increase or decrease in the amount of purin material formed from the nucleic acid of the nucleus.

If the increased elimination of uric acid in dogs injected with toxic sera is the result of altered uric acid equilibrium of the cell, as just described, one would expect to find that, as more xanthin the hypoxanthin are oxidized to uric acid, less of these purins should be eliminated in the urine. On the other hand, if the uric acid is the result of a new formation from the nucleic acids then an increase in purins also would be expected and at the same time an augmentation in elimination of phosphoric acid in some form[10] as the result of the splitting of the nucleic acid molecule. In the formation of uric acid by the first process no change in phosphoric metabolism should take place.

Methods.—The experiments presented in Tables I, II and III were carried out in the following manner: The animals were placed upon nitrogenous equilibrium after which estimations were made during a three day fore period. An injection was then given, and in two instances a second injection after a lapse of one and three days respectively. The observations were continued until after the maximum effect was reached. The purin-free diet was a casein,

[9] Austin, A. E., The Uricolytic Enzyme in Animal Organs, *Jour. of Med. Research*, 1906, xv, 309. The Uricolytic Enzyme, *ibid.*, 1907, xvi, 7.

[10] Jackson, H. C. and Blackfan, K. D., Action of Certain Drugs on the Elimination of Uric Acid During a Nitrogen-free Diet, *Albany Medical Annals*, 1907, xviii, 24.

cracker dust and lard mixture. The urine was examined for total-nitrogen by the Kjeldahl-Gunning method, the uric acid and purin bases were determined by the Salkowski procedure, and the inorganic phosphates by titration with uranium nitrate, using potassium ferrocyanide as an indicator. The method used for total phosphorus was fusion of the evaporated residue of the urine with sodium hydroxide and potassium nitrate, precipitation with ammonium molybdate in the presence of ammonium nitrate, solution of the ammonio-phosphomolybdate in ammonia and re-precipitation with magnesium mixture. This precipitate was filtered off, incinerated and weighed as magnesium pyrophosphate.

The question of the presence of phosphorus in the urine in organic form has recently received attention from various investigators among whom Bergmann[11] and LeClerc and Cook[12] incline to the opinion that all of the phosphorus of the urine exists in the inorganic form. The latter observers studied the phosphorus content of the urine by means of determinations made by Neumann's[13] method, and by that outlined above, and compared the results with those obtained by the uranium-titration procedure. They show that the results by the dry fusion method are uniformly higher by three to four per cent. than those given either by uranium acetate or by Neumann's method; hence they incline to the view that this difference is within the limits of error and that no organic phosphorus is present in normal urine.

We cannot agree with these conclusions in view of the results obtained by one of us in a study of the elimination of organic phosphorus after the administration of sodium salicylate.[14] Nor do they receive support from the results which are presented in this

[11] Bergmann, W., Ueber die Ausscheidung der Phosphorsäure beim Fleisch- und Planzenfresser, *Arch. f. exper. Path. u. Pharm.*, 1901, xlvii, 77.

[12] LeClerc, J. A., and Cook, F. C., Metabolism Experiments with Organic and Inorganic Phosphorus, *Jour. of Biol. Chem.*, 1906, ii, 203.

[13] Neumann, A., Einfache Veraschungsmethode (Säuregemisch-Veraschung), *Zeit. f. physiol. Chem.*, 1902, xxxvii, 115.

[14] Jackson, H. C. and Blackfan, K. D., Action of Certain Drugs on the Elimination of Uric Acid During a Nitrogen-free Diet, *Albany Medical Annals*, 1907, xviii, 24.

communication. The constant uniformity of difference shown by the fusion method over that of titration seems to indicate that the difference is not due to the factor of error in the method. We have performed in this connection some preliminary experiments with a view to explaining the differences reported and expect to continue them more in detail. At present we can say that Neumann's method apparently yields results which agree closely with those obtained by titration with uranium nitrate, using potassium ferrocyanide as indicator. When the fusion method is employed the figures obtained are uniformly higher than by both of the other methods. At present, therefore, we incline to the opinion that phosphorus in other than inorganic form is present in normal urine.

TABLE I.

Nuclein Metabolism. Dog 59.

Date.	Grams Nitrogen as			Grams P_2O_5.		Notes.
	Total.	Uric Acid.	Purin Bases.	Inorganic.	Organic.	
Apr. 10	7.86	0.0015	0.001	0.899	0.147	
11	5.51	0.0159	Lost	0.652	0.093	
12	4.70	0.0141	0.0082	0.690	0.087	
13	4.49	0.0121	0.0097	0.576	0.050	Injection 10 A.M. toxic serum; dose 1:785.
14	6.97	0.0126	0.0231	1.009	0.085	
15	12.50	0.0246	0.0169	0.690	0.121	Injection 11 A.M. toxic serum; dose 1:1745.
16	11.08	0.0127	Lost	0.848	0.072	Hburia.
17	13.81	0.0319	0.0287	0.629	0.083	
18	11.93	0.0203	0.0265	0.866	0.023	Killed.

TABLE II.

Nuclein Metabolism. Dog 61.

Date.	Grams Nitrogen as			Grams P_2O_5.		Notes.
	Total.	Uric Acid.	Purin Bases.	Inorganic.	Organic.	
Apr. 22	3.33	0.0042	0.0086	0.478	0.078	
23	3.83	0.0063	0.0072	0.554	0.052	
24	3.12	0.0050	0.0079	0.584	0.097	Injected 12 M. toxic serum; dose 1:937; vomited.
25	4.57	0.0167	0.0103	0.549	0.085	Hburia.
26	5.74	0.0336	0.0107	0.387	0.074	Hburia.
27	6.83	0.0241	0.0124	0.387	0.084	
28	6.54	0.0200	0.0235	0.326	0.046	
29	5.69	0.0112	0.0086	0.356	0.118	Killed.

TABLE III.

Nuclein Metabolism. Dog. 63.

Date.	Grams Nitrogen as			Grams P_2O_5.		Notes.
	Total.	Uric Acid.	Purin Bases.	Inorganic.	Organic.	
May 14	4.74	0.0057	0.0061	0.520	0.062	
15	4.41	0.0054	0.0068	0.557	0.109	
16	4.26	0.0068	0.0076	0.550	0.072	Injected 11 A. M. toxic serum; dose 1 : 835; vomited.
17	5.29	0.0249	0.0110	0.641	0.062	
18	4.24	0.0143	0.0088	0.508	0.078	
19	4.34	0.0109	Lost.	0.535	0.066	Injected 11 A. M. toxic serum; dose 1 : 557; vomited.
20	4.75	0.0133	0.0181	0.576	0.066	Hburia.
21	7.64	0.0141	0.0116	0.435	0.090	Hburia.
22	8.38	0.0095	0.0187	0.440	0.075	Hburia.
23	6.21	Lost.	0.0133	0.550	0.111	Killed.

Results.—From the figures in the above tables it is evident that the increase of uric acid after injection, as noted in the study of the nitrogenous metabolism,[15] is constant and that there also occurs an increase of purin bases and with one exception of phosphorus pentoxide. This general increase occurs not only after the first but also after subsequent injections. It reaches its maximum on the second day and then falls away. The uric acid increase is greater proportionately than that of the total nitrogen, hence an augmentation of the percentage uric acid in terms of total-nitrogen takes place. The uric acid nitrogen, however, returns to the normal sooner than the latter. The same facts hold true for the purin bases the elimination of which runs parallel, in a general way, to the uric acid output. In the one instance (Table II, Dog 61) in which the phosphorus pentoxide elimination remained constant there still occurred the markedly increased uric acid and purin base output. That the phosphorus pentoxide elimination did not follow that of the uric acid and purin bases is to be explained by the fact that after the injection the animal ate only a quarter of the daily food allowance. Although on the day following the injection the

[15] See third paper of this series "Nitrogenous Metabolism" in this number of the *Journal.*

amount ingested of phosphorus pentoxide was therefore diminished, the elimination of inorganic phosphates remained unchanged. This would indicate that an actual increase in endogenous phosphorus pentoxide formation took place, thus bringing the apparent exception in agreement with our other observations. The organic phosphorus elimination was unaltered.

The question as to the cause of the increased output of uric acid during a purin-free diet, observed after various experimental procedures, has been variously explained. Beebe[16] concludes that the increase found after alcohol administration is to be attributed to a diminished oxidative power on the part of the hepatic cells although the purin bases were likewise increased. In commenting upon this view Jackson and Blackfan, from results which they obtained with alcohol, inclined to the opinion that the facts would warrant rather the assumption that the alcohol causes an increased new formation of uric acid and purin bases, and they adduced as evidence, among other factors, the augmented excretion of organic phosphorus.

According to the view of Schittenhelm, concerning the probable way in which the uric acid is formed from the nucleo-proteid, we would expect, if for any cause oxidization in the hepatic cell is diminished, the xantho-oxydase would be affected, and as a result less uric acid would be formed from the purin bases than under normal circumstances. An increase in purins would also occur if the hydrolytic splitting enzymes were unaffected. An augmentation of uric acid would take place either as the result of a diminished power of the uricolytic enzymes or as the result of an increased new production, other things being equal. In the latter case the purin would also be increased.

The results reported in the paper on nitrogenous metabolism do not indicate that the injection of a hæmotoxic serum causes a prolonged or decided decrease in the oxidative power of the hepatic cell. As a whole the organ continues to perform its functions in the normal way. We must therefore explain the increase in purins and uric acid as a decomposition of nuclear material in the autolysis occurring in the areas of necrosis. This is brought about by

[16] Beebe, S. P., The Effect of Alcohol and Alcoholic Fluids upon the Excretion of Uric Acid in Man, *Amer. Jour. of Physiol.*, 1904, xii, 13.

the hydrolysis of the nucleic acids. Such an explanation agrees with the feeding experiments performed by Sweet and Levene[17] upon a dog with an Eck fistula. They found the ingestion of nucleic acids to be followed by a rise in uric acid and total phosphorus elimination. The fact that the increased excretion of these nuclear compounds usually reaches its maximum in our experiments on the second day, when the autolysis is at its height, would also strengthen this explanation.

The increased elimination of inorganic phosphates which accompanies the output of uric acid would likewise point to the new formation of the latter from the nucleic acids formed as a step in the autolysis of nuclear material. The phosphoric acid radical of these acids is evidently excreted in inorganic form and thus the mechanism differs from that observed in connection with the administration of alcohol and salicylic acid under which circumstances the phosphoric acid is apparently eliminated in organic combination, since no increase in inorganic phosphates occurs.

Allantoïn.—In the dog, the largest part of the quantity of uric acid ingested, as shown by Swain,[18] disappears and is not excreted. In the experiments outlined above it is not improbable, therefore, that a much larger amount of uric acid was produced in the necrosis of the cell nucleus than was eliminated. Of the total amount formed only a small proportion escapes hydrolysis and appears in the urine. Swain also has shown that the ingestion of large amounts of uric acid is followed by the elimination of small quantities of allantoïn.

In order to obtain evidence upon this point the urine of the six animals under observation in the experiments reported in the paper on nitrogenous metabolism, was examined for allantoïn according to the method of Loewi.[19] The results are not uniform but indicate that usually the increase of uric acid is accompanied by an increase in allantoïn; for example, in one the amount of uric acid the day

[17] Sweet, J. E., and Levene, P. A., Nuclein Metabolism in a Dog with Eck's Fistula, *Jour. of Exper. Med.*, 1907, ix, 229.

[18] Swain, R. E., Formation of Allantoïn from Uric Acid, *Amer. Jour. of Physiol.*, 1901, vi, 38.

[19] Loewi, O., Beiträge zur Kenntnis des Nucleinstoffwechsels, *Arch. f. exp. Path. u. Pharm.*, 1900, xliv, 20.

before injection was 0.018 gram and of allantoïn 0.319; in the urine of the twenty-four hour period following the former rose to 0.337 and the latter to 0.669.

CONCLUSIONS.

In necrosis of the liver of the dog produced by hæmotoxic immune sera, the increased excretion of uric acid, purin bases and inorganic phosphorus pentoxide is the result of the hydrolysis of nuclear material occurring during the autolysis of the necrotic tissue.

EXPERIMENTAL LIVER NECROSIS. V. THE FATS AND LIPOIDS.[1]

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The results outlined in this communication constitute a partial report of a somewhat comprehensive investigation[2] now in progress of the chemical processes concerned in the variations occurring in the amounts of fats and lipoids in the hepatic cell under normal and various pathological conditions.

It has seemed advisable, in connection with the other investigations of liver necrosis here presented, to discuss at this time only that part of the general study which deals with the fatty changes in hepatic necrosis brought about by the injection of hæmotoxic immune serum.

The study of such lesions is of peculiar value in view of the attempts which have been made to bring into a relation of cause and effect the autolysis of the organ and the appearance of the fat. We have therefore with this point in view attempted to determine whether in the necrosis (autolysis) which follows the injection of the serum there occurs any alteration in the fat content correspond-

[1] Conducted under grants from the Rockefeller Institute for Medical Research. Received for publication July 11, 1907.

[2] This investigation, including a study of the fatty changes occurring in various experimental lesions of the liver of the dog and of certain pathological conditions of the human liver, will be published later in full by H. C. Jackson and L. K. Baldauf. We wish to express here our indebtedness to Dr. Baldauf for the privilege of utilizing in this partial report that portion of his work which refers to necroses of the liver produced by hæmotoxic serum.

ing to changes observed in the nitrogenous constituents of the cell. Especial interest is attached to this question in view of the somewhat widely divergent opinions held as to the origin of the fat which appears in the so-called fatty transformation of various organs. That the fat does not arise from a peculiar decomposition of the proteid molecule in the cell, the fatty degeneration of Virchow, seems fairly well established. On the other hand, Rosenfeld[3] and others hold that the fat makes its appearance as a simple infiltration from without when for any reason the cell has received an injury which seems to inhibit its oxidizing power. The appearance of fat in organs during phlorhizin poisoning is thus explained by Lusk.[4] Waldvogel,[5] however, who has investigated this question most thoroughly believes that the process is one closely allied to autolysis. His theory is that normally certain substances, which may be called combined fats, such as the ovovitellin of Hoppe-Seyler, or the lecithalbumin of Liebermann, hold the fatty radical in a combination which does not react to microchemical fat stains, such as Scharlach R or Sudan III, and which cannot be removed chemically by the ordinary fat solvents. These substances, however, during autolysis become split in such a manner that the fat radical is liberated in the form of protagon, jecorin, lecithin and even neutral fats. He supports this contention by experiments upon the livers of phosphorus-poisoned animals and upon normal livers undergoing autolysis, in which he has shown there is a marked augmentation during autolysis of such substances as protagon, jecorin, cholesterin, fatty acids and neutral fats. The lecithin, on the other hand, is diminished.

[3] Rosenfeld, G., Fetbildung, *Ergebnisse der Physiologie*, 1902, i, 651; *ibid.*, 1903, ii, 50.

[4] Ray, W. E., McDermott, T. S. and Lusk, G., On Metabolism During a Combination of Phosphorus Poisoning and Phlorhizin Diabetes, *Amer. Jour. of Physiol.*, 1900, iii, 139.

[5] Waldvogel, Autolyse und fettige Degeneration, *Virchow's Arch.*, 1904, clxxvii, 1. Phosphorvergiftung und Autolyse, *Deut. Arch. f. klin. Med.*, 1905, lxxxii, 437. Waldvogel and Mette, Die Autolyse in Menschlichen fettige degenerierten Organen, *Münch. med. Woch.*, 1906, ix, 402. Waldvogel, Die durch Fermente bewirkten Umwandlungen bei der fettigen Degeneration, *Zeit. f. physiol. Chem.*, 1904, xlii, 200.

Siegert[6] has also shown that, although in autolysis the ether extract of the liver does not increase, a marked rearrangement of the fatty compounds of the extract takes place, and that the jecorin rapidly suffers decomposition. This is not in accord with Waldvogel's results. In this connection it may be mentioned that Taylor[7] has conducted experiments upon normal and phosphorus-poisoned frogs and finds that, although the absolute amount of free fat may not increase after the administration of phosphorus the combined fats estimated after digestion with pepsin-hydrochloric-acid suffer a marked diminution equivalent to two thirds of the amount originally present.

As stated in previous papers in this series phosphorus poisoning does not seem to set up in the liver processes which are strictly analogous to autolysis following necrosis, hence the results to be presented must not be considered as strictly comparable to those obtained in other lesions.

Few attempts have been made to study the effect of a true necrosis upon the fat constituents of the cell. Dietrich,[8] as the result of histological studies, claims that autolysis is not an important factor, since tissues introduced into the peritoneal cavity in collodion sacs do not show fatty change. When the tissue, however, is not enclosed in sacs, fat droplets appear which are present not in the cell substance but in the interstices of the tissue. He also tied off the renal arteries and found a "deposition" of fat around the necrotic areas. He believes, therefore, that fat will not appear if the cell is completely dead as in necrosis; but only when it continues to functionate incompletely, as, for example, must be the case with the cells around necrotic areas.

This opinion we can confirm as the result of the histological study of focal necroses caused by hæmotoxic serum. Frozen sections of formalin-hardened material stained with Scharlach R never show more fat, and usually less, than the surrounding adjacent

[6] Siegert, F., Das Verhalten des Fettes bei der Autolyse der Leber, *Beiträge zur chem. Physiol. u. Path.*, 1902, i, 114.

[7] Taylor, A. E., On Fatty Degeneration, *Jour. of Med. Research*, 1903, ix, 59.

[8] Dietrich, A., Experimente zur Frage der fettigen Degeneration *Münch. med. Woch.*, 1904, li, 1510.

normal liver tissue. There is always present, however, in lesions twenty-four to forty-eight hours old a very definite and striking accumulation of fat in the ring of more or less degenerated cells lying between the necrotic and normal liver. These cells correspond to those which in hæmatoxylin and eosin preparations present a vacuolated, granular protoplasm and pycnotic, poorly staining, nuclei.[9]

Di Cristina,[10] who conducted experiments somewhat after the nature of those of Dietrich, and made chemical analyses by Rosenfeld's method, states that no increase of fat occurs in the necrosis caused by shutting off completely the renal circulation. Of considerable interest in connection with Dietrich's view are the experiments reported by Bainbridge and Leathes.[11] These investigators ligated the hepatic artery alone and thereby obtained an increase in fat but no necrosis. The ligation of the portal vein on the other hand resulted in atrophy of the cells and some necrosis, but no augmentation of fat. These experiments appear to confirm the idea that the cells must retain in part their normal function and be normally bathed with the circulating fluids in order to give rise to the appearance of fat within them.

In concluding this brief discussion which merely suffices to indicate the trend of opinion in regard to the subject under investigation it may be said that much of the discrepancy in the results reported can be safely ascribed to the varying methods employed especially in connection with the extraction of the fatty material from the tissue. Siegert has also emphasized the ease with which the extracted products undergo laboratory changes.

Methods.—The organs were removed from the body quickly, put through a hashing machine, weighed, and dried under absolute alcohol at about 70–80° C. At this stage the partially dry material was weighed and then ground in a machine to an impalpable powder. Part of this was further dried in a desiccator to constant

[9] Pearce, R. M., Regenerative Changes in the Liver: A Study of Experimental Lesions in the Dog, *Jour. of Med. Research,* 1906, xv, 99.

[10] Di Cristina, Die chemischen Veränderungen bei fer fettigen Degeneration in Beziehung zur den anatomischen, *Virchow's Arch.,* 1905, clxxxi, 509.

[11] Bainbridge, F. A. and Leathes, J. B., The Effect of Arterial or Venous Obstruction upon the Nutrition of the Liver Cells, *Biochem. Jour.,* 1906, ii, 25.

weight and upon this was calculated the dry substance and nitrogen content of the original tissue. Another weighed part was extracted in a Soxhlet with alcohol and chloroform successively according to the method of Rosenfeld. Each total extraction with alcohol and with chloroform lasted on an average thirty hours. In some cases the original partly-dried material was so fatty that a rough extraction with chloroform at room temperature preceded the grinding. This extract was added to the subsequent one obtained from the Soxhlet. The total fat was taken up in a definite volume of chloroform. An aliquot portion of this was evaporated to complete dryness at about 70° C. and from this was calculated the fat per cent. of the tissue.[12]

For our purposes it was not thought necessary to keep separate for analysis the alcohol and chloroform extracts as Waldvogel does with alcohol and ether. This procedure is exceedingly time consuming and we have employed another and simpler method which we believe has given results equally definite. Instead of attempting to decide whether the fat compounds present in the extract underwent any change or rearrangement, such as is described by Waldvogel during autolysis of normal tissues and in phosphorus poisoning, it seemed sufficient to determine the nitrogen and phosphorus pentoxide content of the extract, and from these figures to calculate the relationship of the nitrogen to the phosphorus. Since the molecule of the lecithins contains one nitrogen and one phosphorus atom the relationship $P:N = 1:1$; in jecorin, however, this ratio is 1 : 4 and in protagon about the same, varying from 1 : 3.4 to 4.8. The latter figure is calculated from the analyses of Dunham.[13] It is seen, therefore, that the greater the preponderance of substances of the jecorin and protagon type in the extract the higher would be the P : N ratio. On the other hand, if these substances should undergo an autolytic change whereby lecithin and fatty acids were

[12] We have employed chloroform in this connection, since, in the first place, it is a much readier solvent for the fatty compounds than either sulphuric or petroleum ether, and secondly, because all of the ether we could obtain reacted distinctly acid to phenolphthalein, a fact already alluded to by Baldauf, Chemistry of Atheroma and Calcification (Aorta), *Jour. of Med. Research*, 1906, xv, 355.

[13] Dunham, E. K., Further Observations on the Phosphorized Fats in Extracts of the Kidney, *Proc. of Soc. for Exper. Biol. and Med.*, 1905, ii, 63.

produced, this ratio should fall to the neighborhood of 1 : 1. Hence from variations in this ratio, changes in the fatty constituents of the extract should be readily determined.

Measured portions of the chloroform extract were analyzed in duplicate as follows:[14]

Total nitrogen by the Kjeldahl-Gunning method; phosphorus pentoxide by the usual fusion procedure and weighing as magnesium pyrophosphate and the iodine equivalent as outlined by the Association of Official Agricultural Chemists.[15] With some exceptions portions of the tissues analyzed were stained with hæmotoxylin and with Scharlach R for the purpose of determining roughly in a comparative way the extent of the necrotic lesion and the fat content.

Results.—The table presents the results obtained in the analysis of four dog livers with normal, four to five, per cent. fat content, one apparently normal but very fatty liver with 21.9 per cent., five with focal necrosis and three with diffuse necrosis of varying degree. As can be seen no relation exists between the degree of necrosis and the amount of fat present. The high amount of fatty material which is present in the normal liver, extractable by the newer method of Rosenfeld, is at first glance surprising, but is in accord with the results of recent investigators. The fat per cent. of all the necrotic livers falls between the normal limits with the exception of 43 and 21 which are above the normal, but these do not represent the most extensive necrosis. Experiments 28 and 29, with the most diffuse necrosis, show normal amounts of fat.

The point which was emphasized in one of the previous papers[16] concerning the percentage of dry substance in the fatty livers is well shown in the table. Whenever the fat per cent. rose above normal the per cent. of dry substance rose almost proportionately. This relation is readily seen by referring to that column in the table in which is given the per cent. of fat-free dry substance. With two exceptions, the dry substance without fat falls between 18.8 to

[14] In most instances the chloroform had to be *completely* removed before the commencement of the analysis.

[15] U. S. Dept. of Agriculture, 1899, Bulletin 46, 50.

[16] See first paper of this series, "The Hexon Bases" in this number of the *Journal*.

TABLE I.

Fats and Lipoids.

Experiment.	Tissue: Per C[redacted]: Dry Substance	Tissue: Dry Substance Fat-free.	Fat.: Moist [redacted]bstance.	Fat.: Dry Substance.	Nitrogen.: Dry Substance.	Nitrogen.: Dry Substance, Fat-free.	Alcohol-chloroform Extra: Per Cent.: Nitrogen.	Per Cent.: P_2O_5.	P:N			
1	25.6	22.6	3.97	15.5	12.7	15.0	—	—	—			
5	28.6	24.1	4.55	15.9	9.7	11.5	1.8	2.6	1:7.0			
2	24.5	19.5	4.95	20.2	12.7	15.9	2.5	5.2	1:4.9			
4	18.5	13.7	4.75	25.7	9.2	11.3	1.5	3.6	1:4.4			
18	40.7	18.8	21.85	53.7	7.5	12.6	0.6	1.3	1:4.5			
7	29.8	26.7	3.10	10.4	9.3	13.2	3.1	7.3	1:4.3			
6	25.3	21.2	4.12	16.3	12.5	16.7	2.4	7.0	1:3.5			
26	28.3	24.5	3.84	13.6	9.9	13.8	2.1	5.0	1:4.3	[illegible]	+	Extensive focal necroses.
43	41.1	24.9	16.23	39.5	8.3	14.0	0.7	2.0	1:3.8	47.0	++	Extensive focal necroses.
27	26.0	20.3	5.72	22.0	11.5	15.5	1.5	6.5	1:2.4	49.3	++	Extensive focal necroses.
21	41.1	14.9	25.15	61.2	6.7	10.5	0.3	1.1	1:3.0	50.0	+++	Diffuse necrosis.
28	23.2	18.8	4.43	19.1	11.9	15.5	2.3	5.8	1:4.3	50.3	+	Diffuse necrosis.
29	21.9	20.1	4.88	19.5	12.7	16.3	2.3	6.1	1:4.0	62.3	+	Diffuse necrosis (most marked)

26.7 per cent. and this surprisingly small variation is in no definite relation to the amount of fat present. The two exceptions are one with normal and one with high fat content. Waldvogel has claimed that as the fatty autolysis increases the water content of the tissue also rises. We would be inclined to ascribe this rather to the nitrogenous autolysis *in vivo* which as we have pointed out elsewhere[16] tends to diminish the amount of dry substance if the circulation is not too greatly impaired. This is shown clearly in Experiments 27, 21, 28 and 29, with the most pronounced necrosis, in which it is seen that the figures for the dry fat-free substance lie on the lower edge of the variation limit for this factor.

As regards the nitrogen content of the tissues a somewhat similar condition of affairs is evident, and although this point does not come directly into this part of the general subject it is of considerable interest. It will be seen that the per cent. nitrogen of the fat-containing dry substance varies within rather wide limits (6.7 to 12.7 per cent.). In those instances, however, where the fat content is high the nitrogen per cent. is low, as would be expected. If, however, the nitrogen of the fat-free dry substance is considered, it is found that the figures for this factor are surprisingly constant throughout (10.5 to 16.7 per cent.). Some of the lowest and highest values occur where, as regards the fat content, the liver tissue was perfectly normal. This indicates apparently that in conditions such as those under discussion, when an increase in fat-content occurs, the material which is represented by the nitrogen of the tissues suffers no decrease or increase in amount. If then the fat originates from some compound proteid antecedent, the proteid component remains apparently unchanged.

A consideration of the character of the fatty extract obtained from normal and necrotic tissues presents some interesting facts. In regard to the iodine equivalent, which indicates roughly the content of oleic acid in the fat mixture, it is evident that as the fat per cent. increases the iodine absorption factor falls below normal (Experiments 18, 43, 27 and 21). This low iodine factor associated with increased fat content would of course point to a diminishing content of oleic acid radicals as the fat is heaped up in the cell. This again is directly opposed to the finding of Waldvogel in phosphorus poisoning.

An examination of the nitrogen and phosphorus pentoxide percentage of the fatty extracts indicates that somewhat wide variations are present, the nitrogen varying from 0.3 to 3.1 and the phosphorus pentoxide from 1.1 to 7.3. It is evident, however, that the lower percentages are in those experiments in which the greatest amount of fat is present (Experiments 18, 43, 27 and 21). With these exceptions the percentage figures are quite regular. Only one explanation can be made of this difference. That is, that as fatty compounds make their appearance in the cell, those substances predominate which do not contain nitrogen or phosphorus, namely the neutral fats, fatty acids and cholesterin.[17]

Of particular interest is the ratio of phosphorus to nitrogen discussed above. It is seen from an examination of the figures in the table that this ratio remains remarkably constant regardless of the absolute variation in the figures. The ratio, with the exception of Experiment 5 which is high, varies only from 1 : 2.4 to 4.9. If we also exclude Experiments 21 and 27 the variation is still further reduced to 1 : 3.5 to 4.9, which is well within the limits of error or better perhaps within the calculation error as made from the published figures for the nitrogen and phosphorus percentages of such poorly defined substances as protagon and jecorin. This high ratio indicates that the nitrogen- and phosphorus-containing fats present in the extracts are of the protagon and jecorin type and remain so irrespective of the amount of fat appearing in the cell.

It must be admitted that some slight evidence does exist to indicate that perhaps under certain conditions these bodies may undergo autolysis. In the two experiments, for example 21 and 27 with a low ratio (1 : 2.4 to 3.0), considerable autolysis must have been going on during the extensive necrosis and the low ratio as a concomitant factor would point to the appearance in the extract of bodies such as the lecithins, which as previously stated possess a ratio of 1 : 1. In these experiments roughly one third of the nitrogen- and phosphorus-containing fats has been replaced by lecithin, according to this reasoning. We are somewhat sceptical, however, of the validity of this line of argument since it does not

[17] We possess confirmatory evidence upon this point in the results which have been obtained with the saponification equivalent. These will be published in a later paper.

agree with Waldvogel's observations; nor is the low ratio present in the two experiments (28 and 29) where one would expect it to be most markedly diminished since the necrosis was most pronounced. In these two instances, however, the ratio is normal. As the low ratio occurs in only two out of the eight experiments in which necrosis was present, we are more inclined to believe that some other factor is the true cause for the change.

SUMMARY.

1. Changes which occur in the fat content of the liver of dogs receiving hæmotoxic serum bear no relation to the degree of necrosis produced by this serum.

2. An increase in water content of the tissue seldom occurs, but where present is due to the nitrogenous autolysis rather than to the deposition of fat.

3. The appearance of fat in the cell is not associated with a decomposition of the proteid component of the compound fats, but rather to a simple splitting off of the fatty radical. This is shown by the slight variations occurring in the percentage nitrogen of the fat-free substance.

4. The iodine equivalent diminishes as the fat content increases. This would indicate that in the fatty changes which occur, fats other than those containing oleate radicals make their appearance.

5. The ratio of phosphorus to nitrogen in the alcohol-chloroform extract remains practically constant in all degrees of necrosis. Hence the substances of the protagon and jecorin type hold the same relation to the lecithins during the autolysis as they do normally.

6. In a general way it may be said that the results obtained in the microchemical staining of the fats with Scharlach R agree with those found by chemical extraction methods.

BIOLOGICAL RELATIONSHIPS OF DIPLOCOCCUS INTRACELLULARIS AND GONOCOCCUS.[1]

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Recent studies by Brickner and Cristéanu[2] having shown a marked similarity between *Diplococcus intracellularis* and gonococcus, as instanced by their agglutinin and precipitin reactions, as well as their effect on inoculated animals, it seemed interesting to follow the same lines of work and carry them further by testing for possible specificity of the immune bodies developed in the sera of animals immunized to these two organisms. Some of the experiments made by Dr. Flexner[3] on the biology of the diplococcus were repeated with the gonococcus in order to detect possible differences.

BIOLOGY OF THE GONOCOCCUS.

Like the intracellularis, the gonococcus is a coffee-bean shaped diplococcus occurring more frequently within leucocytes than outside them. Comparison of smears from the pus of a recent case of gonorrheal vaginitis and from the cerebro-spinal fluid of an early case of cerebro-spinal meningitis (both in infants) shows a marked similarity, in that both present many polymorphonuclear leucocytes containing from one to ten or more pairs of Gram-negative diplococci, and some pairs of cocci lying extra-cellular. There is, however, a decided difference in the size of the two varieties of organisms, the intracellularis being much the larger of the two. This relatively larger size is also seen in smears made from the peritoneal exudate in guinea-pigs killed by inoculations of diplococcus and gonococcus respectively. In agar cultures, twenty-four hours old, on the other hand, the gonococcus is larger, possibly because its growth is so much less profuse.

[1] Received for publication July 2, 1907.

[2] Brickner and Cristéanu, *Compt. rend. de la Soc. de Biologie*, 1906, lx, 846; 942; 988; 1070.

[3] Flexner, *Jour. of Exper. Med.*, 1907, ix, 105.

Gonococcus grows best upon glucose-serum-agar, prepared by adding about one third its volume of human pleuritic or ascitic fluid to the melted agar. The reaction of the medium proved unimportant unless very alkaline to phenolphthalein, a faintly acid, neutral, or faintly alkaline medium giving about equal growths.

The cultures of gonococcus studied were isolated from cases of vaginitis occurring in young infants at the Babies' Hospital. Smears from the discharge in such cases show the gonococcus to be the only organism present in almost every instance, so that glucose-serum-agar plates made from the pus obtained by passing a platinum loop into the vaginal canal gave, in the majority of instances, an abundant and almost pure growth of gonococcus colonies.

Glucose-agar and dog's serum-agar proved useless as a medium for the cultivation of the first generation of gonococci, but subsequent plants from human serum-agar to these media gave a faint growth consisting of separate colonies surviving from two to five days, and bearing transplantation for from two to six generations only. Sheep's serum-agar proved an excellent culture medium, providing that fully one third volume of serum was added. The sheep's serum-glucose-agar ordinarily used for the growth of *Diplococcus intracellularis* was useless, because it contained too little serum. Thalmann's[4] agar gave excellent growths of all the strains of gonococci, but the cultures survived only from seven to twelve days. Picker[5] found that not all strains of gonococcus grew on Thalmann's agar, but some survived twenty-one days, and some even as long as six months, if the tubes did not become dry. The advantage of Thalmann's agar would seem to lie in its reaction, obtained by neutralizing two thirds of the quantity of agar to phenolphthalein and then adding the other, acid, third. Vannod[6] found that plain agar made slightly alakaline to litmus paper is a very suitable medium for the gonococcus, while if the agar is alkaline to phenolphthalein the organism will not grow upon it. I did not repeat Vannod's experiments.

On human serum-glucose-agar slants gonococci remained viable frow twenty-six to thirty-eight days when capped with rubber and

[4] Thalmann, *Cent. f. Bakt.*, 1900, xxvii, 828; 1902, xxxi, 678.

[5] Picker, *Wien. klin. Woch.*, 1906, xix, 1282.

[6] Vannod, *Cent. f. Bakt.*, 1906, xl, 162; 1907, xliv, 10.

kept at 37° C., or as long as the tubes remained moist. The corresponding uncapped cultures in the thermostat usually survived from sixteen to twenty-one days. The addition of a drop of a suspension of calcium carbonate did not prolong the viability of the gonococcus beyond the period of survival on moist serum-glucose-agar, while the danger of making the medium too alkaline with the carbonate is a drawback to its employment.

Gonococcus did not grow in sheep's serum-water containing sugars, but in human serum-water litmus medium to which dextrose or maltose had been added gonococci caused slight reddening without coagulation. Lactose, saccharose, mannite and dextrine were unaffected. In their ability to ferment sugars, gonococcus and intracellularis acted alike. Dunn and Gordon[7] found that the gonococcus did not affect maltose, thus differentiating the two varieties of cocci. The ten strains I isolated from infants all fermented maltose.

Some of the experiments in viability and autolysis made by Dr. Flexner[8] with diplococcus were repeated with gonococcus, suspensions being made in salt solution and also in Ringer's fluid, a duplicate series kept in the ice chest and in the thermostat at 37° C. The suspensions were of four different strengths: the original turbid suspension, and the same diluted twice, five and ten times. The results were almost parallel with those obtained by Dr. Flexner with the *Diplococcus intracellularis,* more cocci remaining viable in the concentrated salt solution suspensions kept at 7° C., and more in the weaker suspensions at 37° C. While in Ringer's fluid the larger number of cocci survived in the concentrated suspension at 37° C., and in the weaker suspension at 7°C. Cover slips were made daily from these tubes, and the cocci found to be less disintegrated in the lower than in the higher salt solution suspensions kept in the thermostat, while in the tubes kept in the ice chest there was remarkably less disintegration, many cocci staining well on the sixth day, although growth had ceased on the third from both salt and Ringer's solution suspensions. Growth took place after six days in sub-cultures made from the salt solution suspensions kept at 37°

[7] Dunn and Gordon, *Brit. Med. Jour.,* 1905, ii, 421.
[8] Flexner, *loc. cit.*

C., and after seven days from the Ringer's solution suspensions. Cover slips showed rather less daily disintegration in the Ringer's solution than in the salt solution. Among the surviving cocci the larger, more resistant pairs, taking on a deep safranin stain, described by Dr. Flexner, were distinctly seen in the cover slips. As in the case of the intracellularis, cold is more injurious than warmth to the gonococcus. But unlike the intracellularis, Ringer's fluid did not prolong the viability of the gonococcus beyond or even up to the period of survival in ascitic-glucose-agar. Even when kept in the ice chest (at 37° C.) growth was obtained in sub-cultures on the seventh day from the serum-agar tubes, while such tubes kept in the thermostat gave excellent sub-cultures on the twenty-seventh to thirty-ninth days. Cover slips showed many deeply staining cocci at that late day, but most of the cocci had been disintegrated.

Two different strains of the diplococcus grew in sub-cultures made on the thirty-fifth day after inoculation on moist sheep's serum-agar, the tubes having been kept constantly at 37° C. A parallel set of tubes kept in the ice chest gave growth for seven days only and no well-staining cocci could be detected in cover slips made on the eighth day. Thus while gonococcus survives longer on solid media kept at 37° C. than does the *Diplococcus intracellularis*, the viability of both organisms on such media at 7° C. is about the same.

As in the case of the diplococcus, the autolytic ferment of the gonococcus is destroyed by exposure to a temperature of 65° C. for thirty minutes. Suspensions in water, salt solution and Ringer's fluid gave the same results in this respect.

PATHOGENESIS OF THE GONOCOCCUS.

Cultures of gonococcus isolated from cases of vaginitis at the Babies' Hospital were inoculated into white mice and young guinea-pigs. The first or second generation was used to inoculate the surface of a pint Blake bottle of serum-glucose-agar. After twenty-four hours at 37° C. the growth was suspended in four cubic centimeters of 0.9 per cent. salt solution and one half injected into the peritoneal cavity of each of two guinea-pigs weighing between 170 and 200 grams. In this way nine different strains were injected in

the second or third generation. All the pigs died within twenty to twenty-four hours. White mice succumbed to smaller doses of recent cultures, one serum-agar slant being sufficient as a rule to cause death over night, half a tube failing to do this. As the mice reacted very irregularly to the inoculations, they were not used extensively.

Cultures lose their virulence readily. It was found that where a second or third generation had caused death in a guinea-pig (170 to 200 grams) in twenty hours, when given doses of half the surface growth of a pint Blake bottle, the sixteenth or eighteenth generation proved not to produce a fatal result when the entire growth in the bottle was injected.

It has been shown by Bail[9] that sub-lethal doses of bacteria may become lethal under the influence of fluids containing aggressins, so-called, which remove the natural protective powers of the organism. While Bail and Weil[10] maintain that aggressins are formed and found chiefly, though not exclusively, in the body fluids, and first at the point of inoculation where the bacteria are proliferating most rapidly, Wassermann and Citron[11] were able to achieve apparently the same results with bacterial extracts made by shaking cultures in normal rabbit's serum or distilled water; and they insist that aggressins are not newly formed in the animal organism, but are merely a dissolved bacterial substance, which is itself toxic. Bail[12] holds the opinion that the natural aggressins described by him, and the artificial ones obtained by Wassermann and Citron are not identical; but into this discussion I shall not enter.

Only artificial gonococcus and diplococcus aggressins, so-called, were used in my work, the extracts being prepared by suspending the twenty-four hours old growth on the surface of a Blake bottle in five cubic centimeters of salt solution, adding a few drops of toluol and leaving the cocci to autolyze over night at 37° C., after which the resulting fluids were preserved in the refrigerator. Just

[9] Bail, *Arch. f. Hyg.*, 1905, lii, 272.

[10] Weil, *Cent. f. Bakt.*, 1906, xli, 121. Bail and Weil, *Cent. f. Bakt.*, 1906, xlii, 51.

[11] Wassermann and Citron, *Deut. med. Woch.*, 1905, xxxi, 1101. Citron, *Cent. f. Bakt.*, 1906, xli, 230.

[12] Bail and Weil, *Cent. f. Bakt.*, 1906, xlii, 51.

before inoculating, the extract was centrifuged until clear or nearly so, and the toluol removed by evaporation in the thermostat (37° C.). Intracellularis extracts were made with ten cubic centimeters of salt solution because the growths were so much more profuse than those of the gonococcus. Whether the addition of this extract to a sub-lethal dose of the coccus would increase its pathogenicity was tested in two ways: First, non-fatal doses of the coccus and its own extract were used; second, the extract of a recent culture (third generation) was given with the cocci of an old culture (forty-ninth generation) and vice versa.

White mice were used for the cross experiments between old and recent strains. It became apparent that the addition of half a cubic centimeter of extract to a non-fatal dose of a twenty-four hour old culture of its own or the other strain of gonococcus caused the death of white mice within twenty-four hours. Specificity of the aggressin is not limited to the homologous strain of gonococcus.

Working with a strain which did not kill a guinea-pig, weighing 170 to 200 grams, in doses of two cubic centimeters, it was found that the addition of a quarter of a cubic centimeter of its extract made the dose a fatal one. Conversely, two cubic centimeters of the extract proving sub-lethal, the addition of a quarter of a cubic centimeter of a surface growth in a Blake bottle suspended in five cubic centimeters of salt solution caused death within ten to twenty hours. But on decreasing the maximum sub-lethal dose of extract or culture in these combinations the animals did not die regularly within twenty-four hours, so that the invariably fatal dose proved to be two cubic centimeters of suspension of the culture plus one quarter of a cubic centimeter of extract, or vice versa.

Working with a diplococcus which was not fatal in doses of half a cubic centimeter (of a ten cubic centimeter salt solution suspension of a twenty-four hour growth on sheep's serum-agar in a pint Blake bottle), the addition of half a cubic centimeter of the extract of the same coccus caused death within eighteen hours, a smaller dose of either extract or culture proving non-fatal. Nothing less than one cubic centimeter of this extract alone killed over night.

The fatal dose of both the gonococcus and the intracellularis combinations having been determined, cross reactions were made. The

suspensions of intracellularis and gonococcus were always made as nearly equal in strength as possible. It was found that a larger dose of gonococcus culture, two cubic centimeters, was required to make half a cubic centimeter of diplococcus extract fatal, while only half a cubic centimeter of diplococcus culture sufficed to make two cubic centimeters of gonococcus extract kill within twenty hours; more was needed than of intracellularis culture to raise the power of the other organism to the fatal point. Thus it becomes evident that the aggressive action of the extracts of gonococcus and diplococcus is more potent for its own than for the other variety of coccus, though the two may act interchangeably in larger doses, and hence are not specific. Dörr[13] has shown the lack of specificity of many bacterial aggressins (coli, dysentery, cholera, pyocyaneus, staphylococcus), and Paul and Lotti[14] found a certain quantitative but not qualitative specificity among them. Bail[15] and Salus,[16] on the other hand, maintain that the natural aggressins are strictly specific.

To prove whether inoculation with living gonococcus cultures or with their extracts protected against intracellularis cultures and extracts, and vice versa, pigs which survived the above experiments were later given a lethal (or larger) dose of the other organism. The diplococcus extract alone, and also non-fatal combinations of extracts and culture did not protect against a fatal dose of a recent diplococcus culture given five to twenty-eight days later. Sub-lethal doses of intracellularis culture (0.05 to 0.2 cubic centimeter) protected against a fatal dose given seven to nine days later, while less (0.025 cubic centimeter) did not protect. The gonococcus culture alone, and the culture plus the extract, enabled pigs to survive a fatal dose of diplococci injected thirteen to thirty days later. Combinations of gonococcus cultures and intracellularis extract protected against a lethal intracellularis dose administered two to five days later. Not only is specificity lacking here, but the gonococcus alone or in combination with its extract seems to be a more powerful

[13] Dörr, *Wein. klin. Woch.*, 1906, xix, 759; 1038; 1081.
[14] Paul and Lotti, *Cent. f. Bakt.*, 1907, xliii, 718; 809.
[15] Bail, *loc. cit.*
[16] Salus, *Wien klin. Woch.*, 1906, xix, 870.

protection against fatal doses of living diplococci than the diplococcus itself.[17]

The anatomical lesions found in guinea-pigs dying within twenty-four to thirty-six hours after inoculation with living gonococcus cultures are very similar to those described by Dr. Flexner[18] in pigs which succumbed to intracellularis injections. There are marked œdema of the pancreas and surrounding tissues, congestion or hæmorrhage of the adrenals, small hæmorrhages into the mesentery, serous coat of the intestines and the parietal peritoneum, with more or less clear or turbid fluid in the peritoneal cavity, and a layer of pus and fibrin over the liver, spleen and omentum. An increased amount of clear fluid in the pleural cavities is often noted. Cover slips from the peritoneum and omentum show varying numbers of polymorphonuclear leucocytes and of diplococci, within and outside these cells. Multiplication of the cocci is more in evidence after inoculation with both culture and extract than when culture alone is injected, and phagocytosis is much less marked under those conditions. When the extract alone has been administered neither cocci nor leucocytes appear in the cover slips, and cultures remain sterile.

SERUM REACTIONS.

Precipitins.—Four sera were tested for precipitin reactions. Three were from rabbits immunized to the gonococcus, and one from a rabbit inoculated ten times with the intracellularis. The sera were tested from twenty to twenty-seven hours after bleeding and the cocci were prepared in four different ways: the sodium hydrate (0.15 per cent.) macerations recommended by Brickner and Cristéanu;[19] salt solution macerations prepared in the same way; salt solution toluol extract described by Flexner;[18] and the filtrate of Thalmann's broth used by Torrey.[20] Cultures of diplo-

[17] The reactions just described call for a special study in order to establish their significance. The failure of the *Diplococcus intracellularis* to induce resistance or immunity, may be due to a slower final recovery period than in the case of the gonococcus. The reaction noted of the gonococcus versus the diplococcus may, possibly be of the nature of the non-specific reactions of resistance produced by such an indifferent body as bouillon which also endure for a brief period of time.

[18] Flexner, *loc. cit.*

[19] Brickner and Cristéanu, *loc. cit.*

[20] Torrey, *Jour. of Med. Research,* 1907, xi, 329.

coccus, gonococcus and *Micrococcus catarrhalis* were used, and normal fresh rabbit's serum as control. Only one serum (from a rabbit receiving nine inoculations of living gonococci) gave any precipitin reaction, and that only in dilutions of one to ten and one to twenty for both gonococcus and diplococcus. It gave no reactions with *M. catarrhalis.* The normal serum and salt solution controls were always negative. Two other anti-gonococcus sera and one anti-diplococcus serum gave negative precipitin reactions, yet all these sera showed the presence of immune body as demonstrated by the deviation of complement (*vide infra*). Muir and Martin[21] have shown that the formation of precipitate is not a necessary accompaniment of the phenomena of complement deviation in antisera. Brickner and Cristéanu[19] found the precipitin reactions with gonococcus and intracellularis in anti-gonococcus serum to be identical. Torrey[22] finds an appreciable difference between the two.

Agglutinins were not high in amount in any serum obtained by immunizing rabbits with increasing doses of gonococcus and diplococcus over periods of eight to ten weeks. Six sera were examined: (*a*) agglutinated both gonococcus and intracellularis in dilutions of one to ten before inoculation, and in one to fifty after seven injections of living cocci; (*b*) agglutinated one to twenty before inoculation and both gonococcus and diplococcus in dilutions of one to four hundred after ten injections of living cocci; (*c*) agglutinated one to ten before inoculation; after ten doses of gonococcus extract, gonococci were agglutinated in dilutions of one to one hundred, diplococci only in one to fifty dilutions; (*d*) agglutinated in one to ten before treatment, ten doses of living gonococci and extract injected, after which both gonococcus and diplococcus were agglutinated in dilutions of one to fifty; (*e*) did not agglutinate either coccus before treatment; eleven inoculations of living diplococci developed agglutinins for both gonococcus and diplococcus in dilutions of one to four hundred, one to six hundred was negative; (*f*) agglutinated in dilutions of one to twenty before inoculation, and after nine doses of living gonococci the serum gave positive agglutination with gonococci in dilutions of one to one hundred, and with intracellularis, one to twenty.

[21] Muir and Martin, *Jour. of Hyg.*, 1906, vi, 265.
[22] Torrey, *loc. cit.*

Bruck's[23] statement that inoculations with living cultures produce a serum rich in agglutinins but poor in amboceptors seems to be borne out in only two of the four sera so produced in my experiments. But the sera showing the highest agglutination reactions were both obtained with living cultures. One of them, however, showed the highest amboceptor content of any serum studied. On the other hand, the sera produced by means of inoculations with extracts with or without living cocci showed very low agglutinations.

The anti-diplococcus serum kindly supplied by Dr. Jobling was from a horse which is being inoculated with cultures and extracts of *Diplococcus intracellularis*. It agglutinated with the intracellularis in dilutions of one to one hundred, and gonococcus, one to fifty.

Brickner and Cristéanu[24] obtained exactly the same amount of agglutination with diplococcus and gonococcus with the serum of a horse inoculated with gonococci. Vannod,[25] on the other hand, found a marked difference in the degree to which these two organisms agglutinated in their respective specific sera, and believed that while group agglutinins exist, a large number of specific ones also exist. Torrey's[26] results led him to the opinion that the agglutinins for gonococcus and intracellularis are common in very low dilutions only. As no one of my sera agglutinated its homologous coccus (grown on Thalmann's agar) in dilutions higher than one to four hundred, the contrast with Torrey's positive reactions in dilutions of one to two thousand to one to seven hundred thousand after nine or ten inoculations is very marked.

Deviation of Complement.—Müller and Oppenheim[27] were the first to demonstrate specific anti-bodies in the serum of a male (adult) case of gonorrheal arthritis by means of the complement deviation test, normal human serum being used as controls.

Bruck showed the presence of specific immune bodies for gono-

[23] Bruck, *Deut. med. Woch.*, 1906, xxxii, 1368.
[24] Brickner and Cristéanu, *loc. cit.*
[25] Vannod, *Deut. med. Woch.*, 1906, xxxii, 1984.
[26] Torrey, *loc. cit.*
[27] Müller and Oppenheim, *Wien. klin. Woch.*, 1906, xix, 894.

cocci in the serum of three adult cases of gonorrhœal disease (two females and one male). Agglutinins and precipitins were lacking in all of them. He also called attention to the existence of such amboceptors in the serum of inoculated rabbits.

Vannod[28] used gonococcus nucleo-proteid for the immunization of rabbits, and obtained a serum which agglutinated gonococcus in dilutions of one to three hundred and contained sufficient specific immune bodies to inhibit hæmolysis by deflecting complement when added in proportion of 0.01 cubic centimeter to the serum. Another serum, agglutinating at one to four hundred, prevented hæmolysis when present in a dilution of 0.025 and 0.001 cubic centimeter. He thinks that agglutinins and specific anti-body develop side by side, not independently, as Bruck[29] has stated.

Four sera were tested for specific immune bodies by means of Bordet and Gengou's[30] method of complement deviation in the presence of antigen, using a hæmolytic system as the indicator. The free receptors in the extract (antigen) having been bound to the complement by the amboceptor (specific) present in the immune serum to be tested, the addition (after one hour) of an inactivated lytic serum and its corresponding corpuscles caused no hæmolysis. In the present tests, washed hen's corpuscles in five per cent. solution and rabbit's serum made lytic to hen's corpuscles were used. Fresh guinea-pig serum was employed as complement, in amounts varying from one twentieth to one fortieth of a cubic centimeter. Normal horse serum and normal rabbit serum were used as controls, as three of the immune sera were from rabbits and one from a horse. The immune sera and the extracts were tested with corpuscles alone and with hæmolytic system alone, with and without complement, before being combined with either.

1. Serum 494, from a rabbit inoculated with gonococcus extract in salt solution. This serum inhibited hæmolysis by deviating complement in dilutions of one to fifty, with one tenth of a cubic centimeter of extract (Table I). The results were identical when diplococcus extract was used. Having found the minimum amount of amboceptor necessary to bind the complement to the receptors in

[28] Vannod, *loc. cit.*

[29] Bruck, *loc. cit.*

[30] Bordet and Gengou, *Ann. de l'Inst. Pasteur,* 1901, xv, 289.

the extract and thus prevent hæmolysis, this amount was doubled and the antigen titrated against it. Then it developed that 0.05 cubic centimeter of immune serum deviated complement in dilutions of antigen from 0.1 to 0.002 of a cubic centimeter (Table II); and, going further by titrating the serum against the smaller dose of extract, 0.005 cubic centimeter sufficed to inhibit hæmolysis in the presence of 0.05 to 0.0005 cubic centimeter of immune serum (Table III). Below this dilution hæmolysis was complete. As the results were identical with gonococcus and diplococcus extracts with anti-gonococcus serum, it follows that the amboceptor in that serum was as readily bound to the pre-receptors in the intracellularis extract as to those in the gonococcus extract, and strict specificity is, therefore, lacking. I am indebted to Dr. Jobling for the suggestion to titrate immune serum and antigen successively against double the smallest binding dose of either. In this way smaller amounts of immune body were demonstrable. It was hoped, also, to bring out specific differences between the effect of the two extracts upon the serum, but none such were obtained.

Normal rabbit serum with similar dilutions of antigen showed no inhibition of hæmolysis. The following tables embody the above results.

TABLE I.

Immune Serum No. 494.	Complement Guinea-pig.	Antigen		Anti-hen Rabbit's Serum.	Hen's Corpuscles.	Results.
		Gonococcus.	D. Intracellularis.			
0.1	0.05	0.1	0	0.01	0.05	—
0.05	"	0.1	0	"	"	—
0.02	"	0.1	0	"	"	—
0.01	"	0.1	0	"	"	±
0.005	"	0.1	0	"	"	+
0.1	"	0	0.1	"	"	—
0.05	"	0	0.1	"	"	—
0.02	"	0	0.1	"	"	—
0.01	"	0	0.1	"	"	±
0.005	"	0	0.1	"	"	+

The sign + means complete hæmolysis; — no hæmolysis; and ± incomplete hæmolysis.

The following controls, nine in number, were made with every series of tests. I give them here instead of repeating them in each

Controls.

No.	Immune Serum.	Guinea-pig Complement.	Antigen.	Anti-hen Serum.	Hen's Corpuscles.	Results.
1	0	0.05	0.1	0.01	0.05	++
2	0.5	0	0.1	0.01	0.05	—
3	0.5	0.05	0	0.01	0.05	++
4	0	0	0	0.01	0.05	—
5	0	0.05	0	0.01	0.05	++
6	0	0	0.1	0	0.05	—
7	0.5	0	0	0	0.05	—
8	0	0.05	0	0	0.05	—
9	0	0	0	0	0.05	—

TABLE II.

Immune Serum No. 494.	Guinea-pig Complement.	Antigen.		Anti-hen Rabbit's Serum.	Hen's Corpuscles.	Results.
		Gonococcus.	D. Intracellularis.			
0.05	0.025	0.1	0	0.01	0.05	—
"	"	0.05	0	"	"	—
"	"	0.02	0	"	"	—
"	"	0.01	0	"	"	—
"	"	0.005	0	"	"	—
"	"	0.002	0	"	"	—
"	"	0.001	0	"	"	+
"	"	0	0.1	"	"	—
"	"	0	0.05	"	"	—
"	"	0	0.02	"	"	—
"	"	0	0.01	"	"	—
"	"	0	0.005	"	"	—
"	"	0	0.002	"	"	—
"	"	0	0.001	"	"	+

TABLE III.

Amboceptor Immune Serum No. 494.	Guinea-Pig Complement.	Antigen.		Anti-hen Serum.	Hen's Corpuscles.	Results.
		Gonococcus Extract.	D. Intracellularis Extract.			
0.05	0.025	0.005	0	0.01	0.05	—
0.02	"	"	0	"	"	—
0.01	"	"	0	"	"	—
0.005	"	"	0	"	"	—
0.002	"	"	0	"	"	—
0.001	"	"	0	"	"	—
0.0005	"	"	0	"	"	—
0.0002	"	"	0	"	"	+
0.05	"	"	0.005	"	"	—
0.02	"	"	"	"	"	—
0.01	"	"	"	"	"	—
0.005	"	"	"	"	"	—
0.002	"	"	"	"	"	—
0.001	"	"	"	"	"	—
0.005	"	"	"	"	"	—
0.002	"	"	"	"	"	+

table. It is hardly necessary to say that only when these controls were correct were the results of the experiments admitted. The quantity of immune serum and antigen varied in each series of controls according to the test to be made. The amount of complement, corpuscles and antigen serum were the same throughout.

2. Serum 495. A second anti-gonococcus rabbit's serum, obtained after ten injections of gonococcus extract and cultures, was found to be anti-hæmolytic without antigen whenever guinea-pig complement was employed. As the serum had been inactivated by heating to 54° C. for thirty minutes, it was thought that anti-hæmolytic substances might have developed under the influence of heat. But on using rabbit complement, hæmolysis was complete, even with 0.2 cubic centimeter of the serum.[31] One tenth cubic centimeter of antigen inhibited hæmolysis in the presence of 0.001 cubic centimeter of immune serum (Table IV). In titrating anti-

TABLE IV.

Amboceptor Immune Serum No. 495.	Rabbit Serum Complement.	Antigen.		Anti-hen Rabbit's Serum.	Hen's Corpuscles.	Results.
		Gonococcus Extract.	D. Intracellularis Extract.			
0.1	0.05	0.1		0.01	0.05	—
0.05	"	0.1		"	"	—
0.02	"	0.1		"	"	—
0.01	"	0.1		"	"	—
0.005	"	0.1		"	"	—
0.002	"	0.1		"	"	—
0.001	"	0.1		"	"	—
0.1	"	0	0.1	"	"	—
0.05	"	0	0.1	"	"	—
0.02	"	0	0.1	"	"	—
0.01	"	0	0.1	"	"	—
0.005	"	0	0.1	"	"	—
0.002	"	0	0.1	"	"	—
0.001	"	0	0.1	"	"	—
1.0005	"	0	0.1	"	"	—

[31] Presumably the guinea-pig serum contained free receptors to which the amboceptor in the immune rabbit serum anchored the complement, thus preventing hæmolysis when the hæmolytic serum was added. On saturating the immune serum with guinea-pig corpuscles over night in the ice chest, and centrifuging the next day, the anti-hæmolytic power of the serum was found to have been lost. The same result was brought about by using rabbit instead of guinea-pig serum as complement.

gen against 0.002 cubic centimeter of immune serum, some differences in the results with the two extracts were noted, the gonococcus being the stronger of the two (Table V).

TABLE V.

Amboceptor Immune Serum No. 495.	Rabbit Serum Complement.	Antigen.		Anti-hen Rabbit's Serum.	Hen's Corpuscles.	Results.
		Gonococcus Extract.	D. Intercellularis Extract.			
0.002	0.05	0.1	0	0.05	0.05	—
"	"	0.05	0	"	"	—
"	"	0.02	0	"	"	—
"	"	0.01	0	"	"	—
"	"	0.005	0	"	"	—
"	"	0.002	0	"	"	—
"	"	0.001	0	"	"	—
"	"	0.0005	0	"	"	—
"	"	0.0002	0	"	"	±
"	"	0	0.1	"	"	—
"	"	0	0.05	"	"	—
"	"	0	0.02	"	"	—
"	"	0	0.01	"	"	—
"	"	0	0.005	"	"	—
"	"	0	0.002	"	"	—
"	"	0	0.001	"	"	±
"	"	0	0.0005	"	"	+

3. Serum 4. A third anti-gonococcus serum was obtained from a rabbit immunized with living cocci. Amboceptor for gonococcus was present in a dilution of one to ten thousand in the presence of one tenth cubic centimeter of extract, and one to two thousand for intracellularis. Table VI gives the results of this and further titrations of the extract.

It is evident that the highest amboceptor content for gonococcus was present in the anti-serum obtained by immunizing a rabbit with living gonococcus cultures, agglutinins being also higher than in the other two sera from rabbits immunized with extracts, and with extracts plus cocci. In these high dilutions a distinct difference between the amount of gonococcus and diplococcus amboceptor was apparent, although the two ran parallel until a dilution of one to two thousand was reached.

The anti-diplococcus horse serum obtained from Dr. Jobling inhibited hæmolysis completely in dilutions of one to five hundred with one tenth cubic centimeter of diplococcus antigen, and incompletely with that amount of gonococcus antigen. Doubling the

TABLE VI.

Amboceptor Immune Serum No. 4.	Guinea-Pig Serum Complement.	Antigen. Gonococcus Extract.	Antigen. D. Intracellularis Extract.	Anti-hen Serum.	Hen's Corpuscles.	Results.
0.1	0.05	0.1	0	0.01	0.05	—
0.05	"	"	0	"	"	—
0.02	"	"	0	"	"	—
0.01	"	"	0	"	"	—
0.005	"	"	0	"	"	—
0.002	"	"	0	"	"	—
0.001	"	"	0	"	"	—
0.0005	"	"	0	"	"	—
0.0002	"	"	0	"	"	—
0.0001	"	"	0	"	"	—
0.00005	"	"	0	"	"	±
0.1	"	0	0.1	"	"	—
0.05	"	0	0.1	"	"	—
0.02	"	0	0.1	"	"	—
0.01	"	0	0.1	"	"	—
0.005	"	0	0.1	"	"	—
0.002	"	0	0.1	"	"	—
0.001	"	0	0.1	"	"	—
0.0005	"	0	0.1	"	"	±
0.0002	"	0	0.1	"	"	+
0.0001	"	0	0.1	"	"	—
0.001	"	0.1	0	"	"	—
0.001	"	0.05	0	"	"	—
0.001	"	0.02	0	"	"	—
0.001	"	0.01	0	"	"	—
0.001	"	0.005	0	"	"	—
0.001	"	0.002	0	"	"	—
0.001	"	0.001	0	"	"	—
0.001	"	0.0005	0	"	"	—
0.001	"	0.0002	0	"	"	—
0.001	"	0.0001	0	"	"	—
0.001	"	0	0.1	"	"	±
0.001	"	0	0.05	"	"	—
0.001	"	0	0.02	"	"	—
0.001	"	0	0.01	"	"	—
0.001	"	0	0.005	"	"	—
0.001	"	0	0.002	"	"	—
0.001	"	0	0.001	"	"	—
0.001	"	0	0.0005	"	"	—
0.001	"	0	0.0002	"	"	±
0.001	"	0	0.0001	"	"	+

inhibitory amount and titrating the two extracts, the results proved to be identical, deviation being complete with 0.02 cubic centimeter of antigen, incomplete with 0.005 cubic centimeter, and absent with a smaller quantity. On testing the inhibitory amount of antigen with diminishing amounts of amboceptor, it was found that 0.01 cubic centimeter was required to prevent hæmolysis with intracellularis, and 0.02 cubic centimeter for gonococcus. Neither the

amboceptor content nor the agglutinins of this serum were high, and the differences for the two varieties of cocci were very small.

Extracts of *Micrococcus catarrhalis,* of *Streptococcus pyogenes,* of a Gram negative diplococcus from a dog, of the typhoid bacillus and of two varieties of dysentery bacilli (Shiga and Flexner types) did not inhibit hæmolysis in combination with this diplococcus immune serum. Normal horse serum controls with gonococcus, diplococcus, streptococcus, *Micrococcus catarrhalis,* and the typhoid and dysentery bacilli were all completely hæmolyzed.

An anti-typhoid serum obtained from Dr. Park at the Board of Health deviated complement in a combination of one tenth cubic centimeter with one two hundredth cubic centimeter of typhoid antigen, but it had no effect upon hæmolysis when combined with intracellularis, gonococcus, streptococcus or *Micrococcus catarrhalis.*

On several occasions extracts of gonococcus and of diplococcus became useless after about two weeks, because they inhibited hæmolysis in doses of one tenth cubic centimeter or less. This was found to be due to the presence of protectin,[32] which fact was demonstrated by shaking the extract with ether for two hours, decanting, evaporating over a water bath and taking up the residue in salt solution. Two tenths and one tenth of a cubic centimeter of this suspension protected blood corpuscles from solution in the presence of their inactivated lytic serum and fresh complement. I am indebted to Dr. Noguchi for this demonstration.

CONCLUSIONS.

The most marked differences, exclusive of pathogenic effects in man, between gonococcus and *Diplococcus intracellularis* are cultural ones, and consist chiefly in abundance of growth and choice of medium.

Relatively larger doses of gonococci than of diplococci are required to kill young guinea-pigs, but the lesions are very similar in the two cases, and both organisms lose pathogenic power rapidly when cultivated artificially.

Agglutinins, aggressins, protective power, and the amboceptors

[32] Noguchi, *Jour. of Exper. Med.,* 1906, viii, 726.

developed in the serum of immunized animals seem to be largely common to both diplococcus and gonococcus.

Neither other Gram negative cocci nor *Streptococcus pyogenes* have any receptors in common with intracellularis and gonococcus.

My thanks are due to Dr. Flexner for suggesting and supervising the work, and to Dr. Jobling for many courtesies.

FURTHER INVESTIGATION UPON THE INFLUENCE OF ORGAN EXTRACTS OF COLD-BLOODED ANIMALS ON THE BLOOD PRESSURE.[1]

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In the spring of 1906, Brown and Joseph,[2] working together, showed that there were either pressor or depressor substances or both present in several organs of various cold-blooded animals. The material collected for that paper was insufficient to make anything more than a very superficial test of the organs used, but during the summer of 1906, Doctor E. P. Lyon, while at Beaufort, North Carolina, and Woods Hole, Mass., kindly collected more material from the same species, and also material not before tested.

The material was preserved in two ways: (1) The fresh organs, such as shark testis, ovary, pancreas and spleen, were cut into thin strips, placed upon filter paper and exposed to the rays of the sun till the water had evaporated and the fat if present in quantity had exuded. The dried material was then wrapped up and shipped immediately to St. Louis, where it was used in a short time. The small amount of oil which remained in the tissue seemed to prevent any deterioration. Before use, this dried tissue was cut into fine pieces and ground up with clean sand in a mortar. (2) Other organs when removed were immediately placed in jars of ninety-five per cent. alcohol, and the jars tightly closed. Before use these organs were put through a meat grinder and ground very fine. They were then spread over a clean glass plate and dried. The alcohol in which the organs had been preserved was evaporated to dryness. The dried residue after this evaporation was added to the dried organ and the whole run through a pulverizer and reduced to a fine powder. No difference in action was observed

[1] Received for publication July 8, 1907.

[2] Brown and Joseph, *Jour. of Physiol.*, 1906, xxxiv.

between the material dried at the seashore and that preserved in alcohol and dried afterwards.

Methods of Preparing Extracts.—The extracts were prepared in various ways. Definite weighed quantities of the dried glands were used in making up the various solutions. Sometimes they were made up to a given percentage in normal saline solution and warmed to 41° C. for fifteen to twenty minutes and then filtered. These were termed "warm extracts." The residue was sometimes made up to its original percentage, either in more normal saline or in distilled water, boiled for from ten minutes to two hours, and then filtered. Such extracts were termed "sediment extracts." Again, a solution was made by boiling a fresh mixture of organ and normal saline for from ten minutes to two hours, then filtering. These were termed "boiled extracts." Extracts were made in ninety-five per cent. alcohol, and also absolute alcohol, filtered, the filtrate evaporated to dryness, and the residue made up to the original percentage in normal saline, so as to make the relative solubilities in physiological salt solution, distilled water, and alcohol apparent by comparing the results obtained upon injecting them into an animal. By the expression "made up to a certain (or original) percentage," I mean this: a three per cent. extract of an organ is one in which three grams of the dried gland were added per hundred cubic centimeters of water. Therefore, a three per cent. extract is the "extract" of three grams of gland in one hundred cubic centimeters of water. In the case of the alcoholic extract, if three grams of gland were added to one hundred cubic centimeters of alcohol, then to the dried filtrate after extraction, would be added one hundred cubic centimeters of physiological salt solution.

Method of Recording Results.—These experiments were all performed upon dogs. The A. C. E. mixture was the anesthetic used in all experiments except when a subcutaneous injection of extracts was made to determine any changes of temperature, in which cases no anesthetic was necessary.

In those cases where tracings of the respiration were taken a "T" tube connected with a tambour was inserted between the tracheal cannula and anesthetic bottle. The blood pressure was recorded by

means of a three-way cannula in the carotid artery, connected with a pressure bottle, and mercury manometer. Sodium citrate (two per cent.) was used to fill the tubing of the blood pressure apparatus. The extracts were injected by means of a burette and a cannula inserted in the central end of the external jugular vein. All injections were made at body temperature. The burette was surrounded by a large glass tube which was kept full of warm water to insure a constant temperature of the injected extracts. In all cases where different extracts were tested upon the same dog, the tubing and burette were disconnected from the venous cannula, and both cannula and burette carefully cleaned out with warm distilled water or physiological salt solution. The moment of injection was recorded by a magnetic signal connected in an electric circuit with the automatic make and break of an induction machine. The time was recorded in seconds by another magnetic signal, and the writing point of this lever was adjusted beforehand so as to show also the line of zero blood pressure. All levers were arranged to write in the same vertical line. Control injections were frequently made by injecting an equal quantity of the physiological salt solution used in making the extracts, but these made it plain that the results obtained when injecting an extract were not due to the salt solution used in making up the extract.

Extracts of the following organs were tested: liver, spleen, pancreas, ovary and testis of shark; liver, kidney, spleen, and testis of swordfish; and spiral valve of dog fish. Care was taken to see that solid particles were absent from the injected substance, except in those cases where the effect of such particles, if present, was under consideration. More than one experiment was performed to determine the different points, but only a single representative effect to show each point will be described. Since the effects of shark and sword fish testes were among the most potent of those examined, an effort was made to determine the following facts regarding them:[8]

I. How small an amount of extract will produce a noticeable physiological effect?

[8] Because of a limited amount of material, however, I have not determined in every case the same point for both shark and sword-fish testis. I might say that in those cases where the same point was determined for each, the results were very similar.

II. Will either the pressor or depressor substance dialyze?

III. Can the pressor and depressor substances be separated:

A. By dialysis?

B. By difference in solubility in absolute alcohol?

C. By difference in solubility in cold normal saline solution?

IV. What is the effect of long-continued boiling?

V. To what physiological change is the marked fall of blood pressure due?

VI. What is the effect of extracts after cutting the vagi?

VII. What is the effect upon respiration?

VIII. What is the effect of subcutaneous injections?

IX. What is the effect of intrastomachic injections?

X. Is the effect of a single extract exactly the same on different animals, or does a personal idiosyncrasy of the animal cause a variation?

Experimental results on these points are as follows:

I. *How small an amount of extract will cause a noticeable physiological effect?*

An extract of shark testis was used in which one cubic centimeter of extract equaled one milligram of the dried gland. An injection of twenty-five cubic centimeters of this extract into a dog weighing eight kilos caused an initial rise of blood-pressure of thirteen millimeters of mercury beginning two seconds after the commencement of injection. In fifteen seconds the pressure had fallen to normal again, and fifteen seconds later was eight millimeters of mercury below normal. The total duration of this depression was thirty seconds. This was the smallest amount of extract used, but it gives a very noticeable though transient effect.

II. *Will either pressor or depressor substance dialyze?*

Method.—An extract of swordfish testis was made as follows: A three per cent. solution of swordfish testis in normal saline was boiled for one hour and forty-five minutes and then filtered through cotton. The filtrate was placed in a dialyzer and dialyzed against a physiological salt solution for five and one half hours.

Results.—Fifty cubic centimeters of the dialysate were injected

Fig. 1. Injection of 50 c.c. of the dialysate from a three per cent. solution from testis of swordfish, dialyzed against 0.9 per cent. sodium chloride for five and a half hours.

(see Fig. 1) into a dog weighing seven kilos. At the beginning of the injection the blood-pressure was 102 mm. of mercury. In twenty-one seconds the pressure had fallen 23 mm. The return of pressure to normal was, for a few seconds, rather rapid, but became slower as it rose, and the normal level was not regained for two and two thirds minutes. There was practically no pressor effect shown. A control injection of an equal quantity of the physiological salt solution used in making the extracts gave neither pressor nor depressor effects. It is, therefore, concluded that the depressor substance will dialyze, but that the pressor very probably will not.

III. *Can the pressor and depressor substances be separated?*

A. *By Dialysis.*

Method.—A three per cent. solution of swordfish testis in 0.9 per cent. sodium chloride solution was warmed at 41° C. for thirty-five minutes, and after standing for some time until solid particles had settled, it was filtered through cotton. The filtrate, which at that time showed the presence of both pressor and depressor substances, was divided into two portions (*a*) and (*b*); (*a*) was put upon ice as it was; (*b*) was put in a parchment tube for dialysis, surrounded by normal saline, and placed upon ice also. Once every twenty-four hours both (*a*) and (*b*) were heated to 76° C. in a water bath, and (*b*) placed in a fresh parchment tube with fresh normal saline surrounding it. This process was kept up for five days, when the extracts were tested.

Results.—(*a*) An injection of twenty-five cubic centimeters of this solution (see Fig. 2) lowered the blood-pressure from 114 mm. to 20 mm. Hg in forty-eight seconds, and it was impossible to get a recovery of the animal. An injection of five cubic centimeters of the same extract into another dog caused the following results: The blood-pressure fell 23 mm. Hg in seventeen seconds, and seven minutes afterward it was not quite back to the level existing at the time of injection. The rate of heart beat just before injection was 115 per minute. One minute afterwards 96 per minute, and four minutes after injection the normal rate of beat had been reëstablished. There was no evidence of the presence of a pressor substance.

(*b*) An injection of fifteen cubic centimeters of this solution ir the same dog used for the latter part of (*a*) above caused a f of 22 mm. Hg, which lasted about four minutes. There was most no evidence of the presence of a pressor substance. The he

FIG. 2. Injection of 50 c.c. of a three per cent. extract of testis of swordf in 0.9 per cent. sodium chloride prepared at 41° C. for thirty minutes.

rate was 158 just before injection, 183 at the point of lowest bloo pressure, and 152 three minutes after injection.

Apparently it is not practical to try to separate the two acti substances in this way, inasmuch as the pressor substance is k or destroyed by the method used. It may have been destroyed the repeated heating to 76° C., the long standing in watery sol tion, or (possibly though not probably) by bacterial action. I not think the cause was bacterial action, since no putrefactive pro esses were detectable. The depressor action of equal doses of (and (*b*) is considerably less prominent in the case of (*b*). Tl is probably the result of removal of a considerable amount of c pressor substance by the long-continued dialysis of solution (*b*).

B. *By difference in solubility in absolute alcohol.*

Method.—It has been found in organ extracts of warm-blood animals,[5] that the depressor substance is soluble in absolute alcoh and the pressor substance insoluble. To see if this would ho

good for these cold-blooded animal organ extracts, solutions were made as follows: A three per cent. solution of sword fish testis in absolute alcohol was made and left standing at room temperature (21° C.) for fifty minutes. It was then filtered through cotton, the filtrate evaporated to dryness and the residue, after evaporation, made up to three per cent. in normal saline. This will be called solution (*a*), and if the results are the same as in the case of warm-blooded animals it should contain depressor and no pressor substance (or at least a less amount of pressor than the normal). The original residue remaining after filtering through cotton was dried, made up to three per cent. in normal saline, boiled for one hour, and then filtered through cotton. This will be called solution (*b*), and it should give pressor and no (or little) depressor effect.

Results.—Without giving exact figures for the effects of these two solutions, I will say that it does not seem possible to separate the two active substances in this way. The pressor effect is about equal in each solution. The depressor action is, however, only half as strong in the absolute alcohol extraction as in the physiological salt solution, seeming to indicate that the depressor at least is less soluble in absolute alcohol than in physiological salt solution.

C. *By difference in solubility in cold normal saline.*

Method.—A four per cent. solution of sword fish testis in 0.9 per cent. salt solution was allowed to stand for seven minutes at room temperature (21° C.) and then filtered. The filtrate was called solution No. 1. The residue was again made up to four per cent. in more normal saline and allowed to stand at room temperature for twenty-five minutes, to see if most of the pressor substance had been dissolved out in solution No. 1. The filtrate this time was called No. 2. The residue made up to the original percentage again, was boiled for some time and then filtered. The filtrate was called solution No. 3.

Results.—The results from this method also are negative. Both substances are found in all the solutions. The depressor substance, however, seems to overshadow the pressor in every case. I believe that the depressor substance is not very highly soluble in water, for I have noticed repeatedly that injections of solutions made up

as (1), (2) and (3) above show almost the same amount of depressor action, and also, that if the length of time that the normal saline is allowed to stand upon the powdered organ is varied, there is not as a rule, a corresponding difference in the amount of depressor action. This is true particularly if the saline is exposed to the organ for longer than about fifteen minutes.

IV. *What is the effect of long continued boiling upon the pressor and depressor substances?*

Method.—A three per cent. solution of swordfish testis was made up in 0.9 per cent. salt solution by warming to 41° C. for thirty-five minutes and filtering. This filtrate was divided into two portions, (1) and (2). No. 1 was left standing unchanged, while No. 2 was boiled for two hours.

Results.—These two solutions, when injected, gave approximately the same amount of fall of blood-pressure, but there was a very marked difference in the length of time the effect lasted in the two cases. Three minutes after injecting No. 1 (see Fig. 3) the pressure had regained a normal level. At the time of injecting No. 2 (see Fig. 4) the pressure was 108 mm. Hg. Immediately following the injection there was the usual sudden fall of blood pressure, but the recovery, though gradual, was exceedingly slow, so slow in fact that twelve minutes after the injection the pressure was only 98 mm. Hg. It was still gradually rising and apparently would have reached the normal level in three minutes more. In No. 2 there was no apparent pressor action, while in No. 1 there was an initial rise of pressure equal to 10 mm. Hg. In neither (1) nor (2) was there any marked effect upon the respiration. The heart rate in (1) was 178 just before injection, 170 at point of lowest pressure, and 174 about two minutes after injection. In No. 2 the rate at time of injection was 123, at point of lowest pressure 101, and twelve minutes after injection 121 beats per minute.

The most apparent thing shown by this line of experiments, is that the pressor substance is rendered inactive by long continued high temperature. The more prominent depressor action following continued boiling of an extract may be interpreted either as a result of the disappearance of the pressor substance alone, or to

Fig. 3. Injection of 50 c.c. of a three per cent. extract of testis of swordfish, showing effect when extract was boiled for twenty minutes.

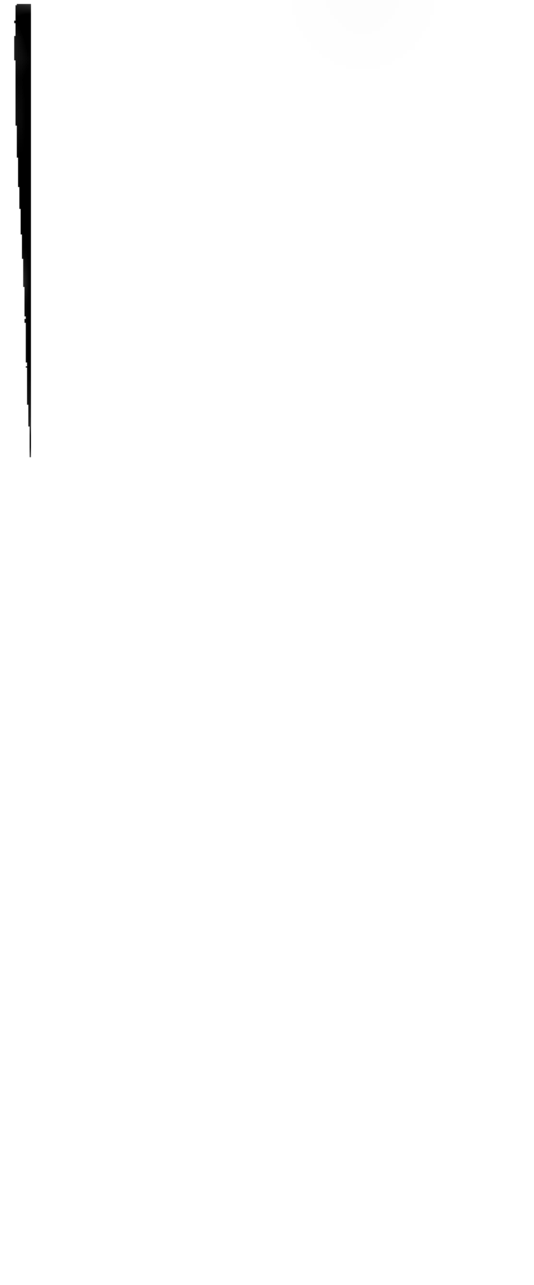

Fig. 4. Injection of a three per cent. extract of testis of swordfish, showing the effect when the extract was boiled for one hour and three quarters. In twelve minutes the recovery was not complete.

that disappearance coupled with the development of another depressor substance not before present.

V. *To what physiological change is the marked fall of blood pressure due?*

Method.—A hind foot and part of the leg of a dog were enclosed in a Mosso's plethysmograph during the time that intravenous injections of extracts were being made.

Results.—An increase in the volume of the enclosed limb followed an injection of twenty-five cubic centimeters of a boiled extract of shark testis. Since in this extract the depressor action is so much more prominent than the pressor, I think it is safe to say that the result obtained, *i. e.,* the vaso dilation was due to the action of that substance. No experiments were performed to determine whether these effects were due to action on the nerve centers, or on the blood-vessels direct.

VI. *What is the effect of extracts after cutting vagi?*

Results.—After cutting both vagi (see Fig. 5) the chief effect noticed is that there is a much more rapid return of pressure to

FIG. 5. Vagi cut; injection of 50 c.c. of a three per cent. extract of testis of swordfish (boiled extract). This injection followed the one shown in Fig. 4 and shows the difference in the curve obtained after cutting the vagi.

normal than when the vagi were intact. The fall is about as great as before, but where normally it took three minutes to recover, it

now takes but little over one minute. The rate of heart beat was little affected either before or after cutting the vagi. I am therefore unable to account for the quick recovery after injecting with vagi cut.

VII. *What is the effect upon respiration?*

Results.—I may say at the beginning that I have been unable to reproduce with these extracts the remarkable results which Doctor Brown and I obtained last year from "Sex-gland of Shark." In the case of which I speak an injection of twenty-five cubic centimeters of extract of sex-gland of shark increased the rate of respiration from forty-eight per minute before the injection to eighty-four one minute after injection. There was also a remarkable increase in the heart rate (from 132 per minute to 264 per minute). I cannot explain last year's results in the light of the effects I have obtained since that time. It was unfortunate that we had so little tissue on hand at the time that work was done. We have been unable to learn either the species or sex of the shark whose sex-gland was examined last year. I have since frequently noticed a slightly increased respiratory rate at the time, after an injection when the pressure was lowest, but I have considered it as a natural result of the low blood-pressure.

VIII and IX. *What are the effects of subcutaneous and intrastomachic injections?*

Results.—I have been unable to get any noticeable effect upon the blood-pressure, heart-rate, or respiration, as a result of injecting by these means. Apparently the absorption is too slow to give any result, or else the substances are changed before reaching the blood-stream.

X. *Is the effect of a single extract the same on different animals, or does a personal idiosyncrasy of each animal cause a variation?*

Method.—A considerable amount of a three per cent. solution of swordfish testis was made up in 0.9 per cent. salt solution and tested upon three dogs.

Results.—In every case the general effect was similar. Individual parts varied slightly. The variation was more marked in the "first" injection in each dog than in the succeeding injections. I also found that where a number of injections were made into

the same dog, the effect of a given dose became less and less pronounced with each injection after the "first" injection. In a few dogs there was a very slight and transient response from the blood-pressure, following the ninth or tenth injection of extract. I had thought that possibly some individual variation due to the animal itself might account for the results which we obtained last year.

The remaining organs were studied only in a preliminary way, and a brief description will be given merely to show their general action.

I. *Shark Organ Extracts:*

(*a*) *Shark Ovary.*—The extracts were made from ovaries containing well developed eggs, and were used in solutions as strong as five per cent. They were tried repeatedly, but no effect worth recording was obtained. This result would indicate that the "Sex-glands of Shark" which Doctor Brown and I used last year was the male organ and not that of the female.

Our records at that time did not show which we were using.

(*b*) *Shark Spleen.*—An injection of twenty-five cubic centimeters of a two and one half per cent. solution of shark spleen in 0.9 per cent. salt solution shows no initial pressor effect. The fall of blood pressure is very rapid, and in this particular case amounted to 54 mm. Hg. The recovery is also very rapid—the normal level being regained in fifty-five seconds after injection. The respiration was not noticeably affected either in rate or depth. The heart rate at commencement of injection was 87 per minute, at the point of lowest pressure 110 per minute, and one minute after injection had slowed down to 104 per minute.

(*c*) *Shark Liver.*—There is nothing particularly interesting to say about extracts of this organ, except that there is a depressor substance present, as in the other organs already described. The effect produced by extracts of this organ is even more transient than in the case of shark spleen. Twenty-five cubic centimeters of a five per cent. solution caused a 42 mm. drop of blood pressure, and in twenty-eight seconds the pressure had returned to its normal level. The respiratory rate was practically unchanged throughout. The heart rate was 132 just before injection, 138 at point of lowest pressure and 156 one minute after injection.

The heart in this case was slower than usual in resuming its original rate.

(*d*) *Shark Pancreas.*—This is one of the most interesting of the organs tested. An injection of twenty-five cubic centimeters of a 2.5 per cent. solution in normal saline (see Fig. 6) caused first an initial rise of blood pressure of 18 mm. Hg, ten seconds elapsing before normal level was regained; second, a fall of 40 mm. which was not recovered from for twenty seconds. In recovering, the pressure did not stop at the normal level, but continued to rise until it was at a height equal to that caused by the initial pressor effect. From this point the pressure gradually fell until in sixty-five seconds it was at the lowest level attained by the first depression. Here the pressure remained (40 mm. below normal) without beginning to rise for over four minutes. The heart rate before injection was 72, during the first depression 90, and during the second depression, two minutes after injection, 66 beats per minute. The rate of respiration before injection was 54, immediately succeeding the injection 66, and two minutes after injection (when the heart rate was 66) 120 per minute. The depth of respiration was almost doubled at the time when its rate was most rapid.

These results might be explained by supposing that there are present two pressor substances and either one or two depressors. If we suppose two pressors and one depressor the order of action would be as follows: The first pressor causes the initial rise, and the depressor action following close upon that of the pressor overcomes the pressor and causes the initial fall. Next the second pressor action shows up and for a few seconds it overcomes the depressor action and causes the second rise in blood pressure. The effect of this second pressor substance is quite transient, and as it wears off the depressor substance resumes its action and holds the pressure at a low level for some time.

II. *Swordfish Organ Extracts:*

(A) *Swordfish Liver.*—Not essentially different from shark liver.

(B) *Swordfish Spleen.*—Not different from shark spleen except that the depressor substance appears to be a little less potent.

FIG. 7. Injection of 50 c.c. of a three per cent. boiled extract of spiral valve of dogfish.

(C) *Swordfish Kidney.*—This organ also contains a depressor substance. It is not, however, potent enough to warrant a definite description of its effects, except, perhaps, to say that recovery from twenty-five cubic centimeters of a three per cent. solution of boiled extract takes over three and one half minutes. The fall of blood pressure is not very great.

III. *Spiral Valve of Dogfish.*

This organ furnishes an interesting extract. It contains both pressor and depressor substances. An injection of fifty cubic centimeters (see Fig. 7) of a three per cent. solution of spiral valve in 0.9 per cent. salt solution (boiled one and a half hours) produced an initial rise in blood pressure of 18 mm. Hg, followed by a fall of 80 mm. Hg. The recovery was very gradual and it took over four minutes to accomplish it. The only difference between the effect of this boiled extract and a "cold" extract, made by allowing the powdered organ and physiological salt solution to stand at room temperature for twelve minutes, is that the depressor action is more permanent in the case of the boiled extract. Recovery occurs in the case of the cold extract in one and a half minutes. The heart rate was 180 just before injection, 210 when the pressure was lowest, and had practically returned to 180 by the time the normal blood pressure was regained.

SUMMARY.

1. None of the extracts examined are very potent in small doses. A noticeable effect, however, is produced upon the blood pressure by the extract of fifteen milligrams of shark testis.

2. A small dose of a five per cent. shark testis extract seems to show more pressor action than a larger dose of the same solution.

3. The fall of blood-pressure is due to a vaso-dilation.

4. After repeated (seven or eight) injections of almost any extract, an injection of more of the same extract, or of another extract, which would be rather potent as a "first" injection, causes very little if any response from the blood-pressure.

5. Continued boiling of an extract destroys the pressor substance.

6. The depressor action is then very pronounced and especially prolonged.

7. The depressor substance will dialyze.

8. The pressor and depressor substances cannot be separated by dialysis, because in the repeated sterilizations the pressor substance disappears.

9. The pressor and depressor substances cannot be separated by absolute alcohol, as each seems to be soluble. The solubility is not, however, very great, at least in the cold.

10. It seems impossible to separate the pressor and depressor substances by means of a difference in solubility in cold physiological salt solution, since the depressor substance is even more soluble in that solution than the pressor. And yet the depressor substance is not nearly all removed by this means, even though it is allowed to stand for some time.

11. After cutting the vagi, the recovery from the effect of the depressor substance is much more rapid than with the vagi intact. The amount of fall of blood-pressure is about equal in either case.

12. Extracts of the testis and pancreas of shark; of the testis and spleen of swordfish; and the spiral valve of dogfish show the presence of a pressor substance.

13. Extracts of the testis, spleen, pancreas and liver of shark; of the testis, liver, spleen and kidney of swordfish, and the spiral valve of dogfish show the presence of a depressor substance.

14. Extracts of shark ovary gave no result. I believe, however, that if sufficient of the parenchymatous tissue of the ovary had been obtained a result would have been given by this organ also.

A Comparison of the Properties of Organ Extracts of Cold- and Warm-blooded Animals.

Cold-blooded Animals.	*Warm-blooded Animals.*
1. Most organ extracts show the presence of both pressor and depressor substances.	Same.
2. The pressor substance is best obtained by extracting with normal saline at the room temperature, but this is not a very successful means of separating the pressor and depressor substances.	Vincent and Sheen,[4] state that this is the best way to *obtain* and *separate* the two.
3. The pressor action is more transient than that of the depressor.	Same.[4]
4. Boiling, if continued for any length of time, destroys the pressor substance.	Same.[4]
5. Long continued boiling causes a more prominent depressor action either by forming new depressor substances or by removing the pressor.	Same.[4]
6. The depressor substance is much more prominent in its action than the pressor and is probably present in larger amounts.	Same.[4]
7. The fall in blood-pressure seems to be due to a dilatation of peripheral vessels.	The fall of blood-pressure is due to a direct action upon the blood-vessels and not to an action upon the vaso-motor center.
8. The effects of "second" and later injections are not the same as those produced by "first" injections.	Same.[5]
9. Both pressor and depressor substances are soluble in absolute alcohol, and they cannot be successfully separated in this way.	According to Schäfer and Vincent,[5] the pressor substance is insoluble in absolute alcohol and the depressor soluble. They used this means to separate the two.
10. The depressor substance is dialyzable.	Same.[5]
11. No effect is obtained by injecting subcutaneously.	Same.[6]
12. The only difference in the effect of injections made before and after cutting the vagi, is that the normal blood pressure is regained sooner after cutting the vagi than it was before cutting.	No difference in the action of extracts injected before and after cutting vagi.

[4] Vincent and Sheen, *Jour. of Physiol.*, 1903, xxiv, 242.
[5] Schäfer and Vincent, *Jour. of Physiol.*, 1899, xxv, 87.
[6] Osborne and Vincent, *Jour. of Physiol.*, 1899, xxv, 283.

THE CHEMISTRY OF THE LIVER IN ACUTE YELLOW ATROPHY.*

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Chemical studies of the liver in true acute yellow atrophy are extremely few in number; of these but one or two have been made since the studies of Kossel and Fischer furnished improved methods for the isolation of monamino and diamino acids, and in no instance has a thorough study of the entire composition of the liver been made. Idiopathic acute yellow atrophy offers us the most striking example of *intra-vitam* autolysis of tissues that occurs in human pathology, excelling even pneumonia in interest because in the latter it is merely an inflammatory exudate that is digested, whereas in acute yellow atrophy the liver tissue itself is destroyed, and often as much as three quarters of the entire parenchyma is removed by autolysis within a few days. Hence we should expect to find the liver showing many interesting chemical changes, especially in regard to the products of the autolytic process. But scanty information on this subject is as yet available. Many years ago Frerichs first detected leucin and tyrosin in the liver in such cases, by microscopic means, and later observers have isolated these substances from extracts of the liver. The only instance found in which modern methods have been used is in the report by A. E. Taylor.[1] This author was able to detect no free arginin, histidin or lysin by the method of Kossel and Kutscher; but by Fischer's ester method he obtained and identified 0.35 gram of leucin and 0.612 gram of aspartic acid. Tyrosin was not isolated. The same author studied an instance of the rather similar form of hepatic atrophy that is occasionally observed as a sequel of chloroform

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[1] *Zeit. f. physiol. Chem.*, 1902, xxxiv, 580; *Jour. of Med. Research*, 1902, viii, 424.

anesthesia, and found in the liver 4 grams of leucin, 2.2 grams of tyrosin, and 2.3 grams of arginin nitrate.

Sootbeer[2] has reported a case of supposed acute yellow atrophy in which a small piece of liver tissue was examined for amino acids with negative results. Beebe[3] investigated two livers, said to be examples of acute yellow atrophy, for their pentose content, but found no decided alteration; if anything the amount of pentose was somewhat larger than usual. Heffter[4] found the amount of lecithin decreased in two livers which he calls, without description, acute yellow atrophy; but it is evident from his analytical figures of the water and fat content that he was dealing with some other condition. Perls and v. Starck[5] found the proportion of water greatly increased in the liver of acute yellow atrophy, a finding corroborated by Taylor and others. They also found the proportion of fat quite similar to that of the normal liver, ranging in their cases from 4.2 to 8.7 per cent. of the weight of the fresh liver, in marked contrast to the 25 to 30 per cent. found in fatty livers. Beyond these few reports there seem to be no analyses recorded in the available literature.

Having the fortune to obtain an autopsy a few hours after the death of a patient with a typical case of this rare disease, thus obtaining the entire liver in a very fresh condition, it seemed desirable to study the material at my disposal in as thorough and systematic a manner as possible. The case from which the material came was a very typical one, of so-called idiopathic acute yellow atrophy, and the anatomical features have already been described elsewhere.[6] Briefly, the chief features were the following:

A young man, aged 20 years, was taken slightly sick without evident cause, the first symptom being jaundice. He gradually grew weaker, and after a week became bedridden. After about four weeks he became unconscious, the jaundice deepened, delirium set in, the area of liver dullness decreased, and there was tenderness in the region of the liver. Death occurred six weeks after the onset of the illness, and two weeks after the illness had become severe. Autopsy was performed three and one-half hours after death, and the chief

[2] *Arch. f. exp. Path. u. Pharm.*, 1903, l, 294.
[3] *Amer. Jour. of Physiol.*, 1905, xiv, 237.
[4] *Arch. f. exp. Path. u. Pharm.*, 1891, xxviii, 97.
[5] Quoted by Quincke, *Spec. Path. und Ther.*, 1899, xviii, 297.
[6] Wells and Bassoe, *Jour. of the Amer. Med. Assoc.*, 1905, xliv, 685.

changes were in the liver, which weighed but 970 grams. The description of the liver follows:

The surface of the left lobe and the margin of the right lobe are decidedly wrinkled, while the upper part of the right lobe is not shrunken and appears much firmer. The left lobe and the margin of the right may be doubled on themselves. On section, the lobules of the shrunken portion are seen to be much smaller than normal, while the lobules of the remainder of the liver tissue are enlarged. The left lobe is of uniform consistence, and is more pliable but not softer than normal liver. The margin of the right lobe is the same except that scattered through it can be felt pea-sized nodules, some of which can be seen protruding on the surface. These nodules are most abundant at the junction of the atrophied and large portions of the liver; in the upper part of the liver these nodules can not be felt. The cut surface of the atrophic portion is of a brownish-red color, becoming lighter yellow in the enlarged portions. The lobules of the large part are enlarged, yellow in color, the centers being darker that the periphery and the cut surface is irregular. The bile ducts are not distended. In the atrophied portion the lobules are very small and of normal color. The small nodules described above on section correspond to islands of yellow tissue lying in the red. The lymph glands at the hilum are enlarged, brownish at the center and pale at the periphery. The *gall bladder* is not enlarged and is of normal size. It contains a very thick green-black bile. No concretions are demonstrable. The mucous membrane appears normal. Bile ducts are all patent.

Microscopically the usual changes of acute yellow atrophy are present. In the yellow areas the parenchyma cells are swollen, stain poorly, and in the center of the lobule have disappeared to a considerable extent; the bile vessels and periportal connective tissues have begun to proliferate. In the red areas the liver cells have entirely disappeared, leaving the very small lobules composed entirely of the original stroma and capillaries, and proliferating cells coming from the bile ducts about their periphery. When stained with Sudan III the absence of fatty changes is striking, only a few of the most atrophied liver cells containing a few minute fat droplets.

Description of Analysis.

Immediately after the autopsy, 700 grams of the liver, chosen in such a manner as to represent fairly all the different portions of the liver in proper proportions, was taken for analysis. It was cut up into thin slices and placed in strong alcohol, sealed, and kept in a dark place until the analysis could be undertaken. One sample was dried to constant weight, and the amount of water present was determined by difference; it was found to amount to 83.8 per cent., there being 16.2 per cent. of solids.

The alcohol-hardened tissue was cut into fine pieces, and extracted repeatedly with boiling 95 per cent. alcohol under a reflux condensor, all extracts being combined. It was then ground fine in a mill, and extracted several times more; this process of grinding and extracting being repeated four times. Finally the material was extracted in a Soxhlet apparatus, jacketed so that the temperature was kept near the boiling point of alcohol, until the extracts were colorless. The residue was then dried, and extracted repeatedly with ether and ligroin until

nothing more was dissolved out; all the extracts being united, and evaporated to dryness *in vacuo,* at a temperature under 45°.

The extracted liver tissue was now repeatedly extracted with water at room temperature, with much shaking; and then with water at 60°. These extracts were united and reduced to one liter, and analysis of a small fraction showed that they contained in all 1.94 grams of nitrogen. They gave a faint biuret reaction. Only traces of ammonia (less than 20 milligrams) could be detected by Folin's method.

For the next extraction of the liver tissue, boiling water was used, and the extraction continued until the extracts were nearly colorless. They were concentrated to 700 cubic centimeters, and, on cooling, the solution gelatinized. The nitrogen content was 1.835 grams and the solution gave Millon's reaction, but not the tryptophan reaction, being apparently nearly pure *gelatin.* As gelatin contains about 18 per cent. of nitrogen there were, therefore, in 700 grams of liver tissue approximately 10 grams of gelatigenous substance, and in the entire liver 13.8 grams; this constituted about 8.8 per cent. of the entire dry weight of the liver substance.

Composition of the Insoluble Residue.—After all extractions had been completed the residue was dried to constant weight, and analyzed. Total nitrogen was determined by the Kjeldahl method and found to be 15.6 per cent., indicating that the residue was very nearly pure protein. The distribution of this nitrogen in the form of monamino, diamino and amid nitrogen was determined according to the Hausmann method, as used by Osborne and Harris,[1] with the following results:

Sample.	*A*	*B*	Ave.
Amid nitrogen.	0.82	0.90	0.86
Melanoid nitrogen.	0.48	0.40	0.44
Diamino* "	4.15	4.11	4.13
Monamino** "	10.10	10.25	10.18
Total	15.55	15.66	15.61

* This fraction also contains part of the purin nitrogen.

** The monamino nitrogen was determined directly in an aliquot part of the filtrate, rather than by difference as is usually done. The large amount of phosphotungstic acid does not prevent successful combustion if sufficient sulphuric acid and time are used.

In view of Wakeman's[2] observation that in phosphorus poisoning of dogs the arginin of the liver seems to decrease more rapidly than the other diamino acids, it was attempted to determine the relative proportions of the diamino acids in the insoluble proteins of this specimen. The method of Kossel and Kutscher was followed. 35.9 grams of the extracted residue was hydrolyzed fourteen hours with sulphuric acid, and the determination of *histidin* made in the usual manner. There was found, in an aliquot part of the histidin precipi-

[1] *Jour. of Amer. Chem. Soc.,* 1903, xxv, 323.

[2] *Jour. of Exper. Med.,* 1905, vii, 292.

tate, nitrogen to the proportion of 0.540 gram, which corresponds to 2.05 grams of histidin. Unfortunately, the filtrate from the histidin, which contained the arginin and lysin, was lost through the breaking of a beaker, so the amount of these two substances cannot be determined. As the original material contained 4.13 per cent. of nitrogen precipitable by phosphotungstic acid, there was left as nitrogen in the solution not over 0.9 gram of nitrogen as lysin and arginin. If human liver protein has the same composition as dog liver protein the proportion of histidin found is relatively high in this specimen, indicating a low proportion of arginin or of lysin, or both.

A portion of this residue was also analyzed for sulphur, phosphorus, iron and ash, with the following percentage results:

	I.	II.	Ave.
Sulphur.	0.80	0.84	0.82
Phosphorus.	0.51	0.49	0.50
Iron.	1.33	1.12	1.22
Ash.	2.0	1.8	1.9

Fats and Lipoids.—All the extracts with alcohol, ether, and ligroin were united, and evaporated to dryness *in vacuo* at a temperature below 45.° (In the distillate there was always a minute amount of a yellow, gummy substance, the nature of which was not determined.) The residue was thoroughly extracted with ether, and then with ligroin, and the extracted material, which constituted but a small part of the entire residue, was freed from ether and ligroin. The ether-soluble material weighed 17.2 grams, had a decidedly acid reaction, and the odor suggested the presence of a trace of acetic acid. It was dissolved in alcohol and the free acids neutralized with one-tenth normal sodium hydroxide solution, of which 35.2 cubic centimeters were required, indicating the presence of 0.92 gram of *free fatty acids* reckoned as oleic acid. The material was dried down with a large volume of sand, and extracted with ether, leaving the salts of the free fatty acids in the sand. The ether extract showed a yellowish green fluorescence, and examined spectroscopically showed the absorption bands of *urobilin.* The ether extract was reduced to 100 cubic centimeters and 20 cubic centimeters were taken for analysis for phosphorus, to determine the proportion of *lecithin.* The amount of phosphorus found in this fraction was 0.0236 gram, indicating the presence of 3.1 grams of lecithin in the entire material, assuming that lecithin contains 3.8 per cent. of phosphorus. This corresponds to a total of 4.4 grams in the entire liver, or 0.45 per cent. of its fresh weight, and 17.6 per cent. of all the ether-soluble material.

The lecithin was removed by precipitating with four volumes of acetone, and the process repeated. The total weight of the solid material left after removing the lecithin, allowing for the fraction taken for determination of phosphorus, was 14.0 grams, and consisted, presumably, of neutral fats and cholesterin. The *cholesterin* was determined by Ritter's method, after saponifying the fats with sodium alcoholate, drying out in a large volume of salt, and extracting with ether. A small amount of sodium alcoholate carried out by the ether, and also traces of glycerin, were removed by shaking out with water, and the cholesterin ob-

tained in a very pure condition by crystallizing out of hot alcohol. The quantity obtained was 1.904 grams, which corresponded to 2.38 grams in the entire material analyzed, or 2.05 per cent. of the dry weight. The free fats, estimated by difference after removing the cholesterin, weighed 11.62 grams, corresponding to 16.2 grams in the entire liver, or 10.3 per cent. of the entire dry weight.

Free Volatile Fatty Acids were sought in the soaps obtained on neutralizing the original ethereal extract, by distilling in acid solution, and collecting the distillate in tenth-normal sodium hydroxide. But 1.1 cubic centimeters of the alkaline solution was neutralized, indicating the presence of mere traces of volatile acids. The free higher fatty acids were isolated by cooling the hot acidified aqueous solution; in this way 0.9 grams of free fatty acids were obtained. *Lactic acid* was sought in the filtrate, but no crystallizable zinc salt could be obtained.

The amount of fat held in the tissue in a form not available to extraction by alcohol and ether was determined by digesting five grams of the extracted residue with pepsin-hydrochloric-acid mixture, and the material thus obtained, after desiccation, was extracted with ether. Only 11 milligrams of ether-soluble material was extracted, indicating that at the most not over 0.3 per cent. of the total dry weight of the liver tissue was combined fat not removable by prolonged alcohol extraction.

Soluble, Non-coagulable Proteins.—These were studied in the hot and cold water extracts. As before mentioned, the hot water extract seemed to consist solely of *gelatin*, and contained 1.835 grams of nitrogen, corresponding to about 10 grams of gelatin. The cold water extract and the extract at 60° C. contained but a minute quantity of gelatin, gave the biuret reaction, and contained 1.94 grams of nitrogen. These extracts were separately evaporated to a small bulk and let stand in the cold, but no crystallization occurred. They were therefore united, evaporated to a thick syrup, and slowly poured into a large volume of 95 per cent. alcohol, to precipitate the proteids. The alcoholic solution was separated by filtration, and the proteins redissolved and reprecipitated four times. The alcoholic filtrates were united, evaporated to dryness, dissolved in water, and slowly concentrated after decolorization with animal charcoal. A small quantity of typical *tyrosin* crystals appeared, which gave the usual tests for that substance and weighed 0.25 gram. From the tyrosin filtrate a small amount of material with a sour flavor, resembling meat extract, crystallized out and this was added to the original alcohol extracts for study later, as was also the mother liquor.

The proteins precipitated by alcohol were dissolved in hot water, cooled, and let dialyze in a collodion sac for six days in the cold (with thymol, toluol and chloroform) against distilled water, which was frequently changed. The diffusate was concentrated, and found to contain but a small quantity of material, which reacted positively to the biuret, Millon's and tryptophan tests. The dialysate solidified into a jelly-like cake inside the dialyzing bag, and gave the Millon's but not the tryptophan test. These two solutions, containing respectively diffusible and non-diffusible proteins, were examined for their contents in proteins, proteoses and peptones, and non-protein nitrogen, according to the method of Bigelow and Cook.[*]

[*] *Jour. of Amer. Chem. Soc.*, 1906, xxviii, 1485.

The diffusate was concentrated, made up to 150 cubic centimeters, and divided into three fractions of 50 cubic centimeters each.

Fraction I analyzed for total nitrogen, gave 0.144 gram, or 0.432 gram for the entire material.

Fraction II was made slightly acid with sulphuric acid. Zinc sulphate was added to saturation; the solution was let stand over night, filtered; the filtrate was washed with saturated zinc sulphate solution, and the proteose nitrogen in the precipitate was determined. I found 0.015 gram, corresponding to about 0.27 gram of *proteoses* in the entire diffusate.

Fraction III 15 grams of salt and 30 cubic centimeters of a saturated tannic acid solution (containing 1 milligram of nitrogen in blank) were added at a temperature below 15°. The solution was made up to 100 cubic centimeters and let stand over night in the cold. 50 cubic centimeters were filtered off and the nitrogen in this fraction, which contained extractives, ammonia compounds, and possibly amino acids, determined. 0.0441 gram nitrogen, or 0.264 gram in the entire solution, was found as non-protein nitrogen. In the tannic acid precipitate there was, therefore, by difference, 0.122 gram of nitrogen in the form of *peptones.*

The dialysate was analyzed in the same way, but equal amounts of nitrogen were obtained in both the zinc sulphate precipitate and the total nitrogen fraction, indicating the total absence of peptones and non-protein nitrogenous substances.

Amino Acids.—These were contained chiefly in the portion of the alcohol extracts that was insoluble in ether. This material was dissolved in hot water, and nitrogen determined in a sample. The result indicated the presence of 4.69 grams of nitrogen in this fraction, which was then studied according to the method followed by Schumm[10] in his study of autolysis in leukemic spleens, certain modifications being introduced as indicated during the work. As Schumm has detailed his methods quite fully, it does not seem necessary to repeat the description in this place. Determination of the nitrogen in the filtrate of the phosphotungstic acid precipitate showed a total of 1.541 grams in forms precipitable by this reagent, which were presumably diamino acids and purins; while 3.148 grams nitrogen was presumably in the form of monamino acids, ammonia compounds, and simple extractives.

The following substances were isolated from the phosphotungstic precipitate:

I. *Free purins,* the total quantity containing 0.069 gram of nitrogen; both *xanthin* and *hypoxanthin* were isolated in characteristic forms (hypoxanthin silver nitrate, xanthin silver), but no guanin or adenin seemed to be present. Quantitative estimation of the purins was not attempted because of the minute quantity of each present, but the hypoxanthin was considerably the more abundant of the two.

II. *Diamino Acids.*—Histidin was present to the amount of 0.64 gram, and *lysin* amounting to 1.04 grams, as estimated from the amount of nitrogen contained in the fractions in which they were present. The lysin was isolated as the picrate, and identified by its crystalline form. *Arginin* could not be isolated, and a determination of nitrogen in the fraction in which it should have been precipitated showed only traces of nitrogen.

[10] *Hofmeister's Beiträge,* 1906, vii, 175.

Monamino Acids.—For the isolation of the monamino acids there was available the filtrate from the phosphotungstic acid precipitate of the original alcoholic extract, and also the non-protein portion of the aqueous extracts which had already yielded 0.25 gram of tyrosin. After removal of the phosphotungstic acid with barium hydroxide in the usual manner the two solutions were united, and the amino acids first sought by simple fractional crystallization. By this means 0.45 gram more of *tyrosin* was obtained. This was identified by means of its typical appearance when crystallizing out, its insolubility in glacial acetic acid, by which it was purified, and by the intense Millon's reaction.

Similarly by fractional crystallization 4.16 grams of approximately pure *leucin* was obtained from several fractions, which, when purified, had a decomposition point of 291°. The copper salt formed from this had the typical appearance of the copper salt of leucin, and contained 19.8 per cent. of copper, the theoretical content of this salt being 19.6 per cent. Nitrogen in the purified product was found to be 10.85 per cent., the theoretical amount for leucin being 10.69 per cent. It is possible that a certain amount of amino-valerianic acid may have been mixed with the leucin, but it was not attempted to make a separation because of the small amount of material.

About two grams of other material also came out on further concentration, which seemed to consist largely of *bile salts;* it also gave Jaffe's reaction for *creatinin,* but the amount was not quantitatively determined.

The solution was then saturated in the cold with hydrochloric acid gas, and a small amount of typical *glutaminic acid hydrochloride* was obtained; this was united with that obtained later by the ester method, and the combined products purified and studied together.

The solution left after these various crystallizations was then esterified three times after the method of Emil Fischer, and the esters were set free and fractionated in the usual manner. The following fractions were obtained:

	Temperature.	Pressure.	Weight of Ester.
1	to 65°	10 mm.	1 gram.
2	65–100	10	2.5
3*	100–120	1–6	1
4	120–190	4–6	1
5	Residue.	—	3.5

* Fractions 3 and 4 were obtained by the use of the mercury pump. As no liquid air condensation was used the gases formed by decomposition at the higher temperatures prevented a successful evacuation, so that pressures less than 4 mm. could not be obtained.

The esters were hydrolyzed in the usual way, and the amino acids separated by fractional crystallization.

From Fraction 1 was obtained 0.2 gram of a white, crystalline material, sweet-tasting, with a melting point of 240°, and containing 18.48 per cent. of nitrogen—calculated for *glycocoll,* 18.67 per cent.

From Fraction 2 was obtained, by precipitation from hot aqueous solution

with alcohol, 0.3 gram of white, rhombic crystals, with a melting point of 274°, a sweetish taste, and containing 15.60 per cent. of nitrogen—calculated for *alanin,* 15.73 per cent. The alcoholic filtrate from the alanin contained, when evaporated, a yellowish, non-crystalline residue, with the odor characteristic of *pyrollidin carbonic acid.* A copper salt was readily formed, which was entirely soluble in absolute alcohol; this salt weighed 0.475 gram, corresponding to 0.354 gram of prolin. The dehydrated salt (dried at 115° until of constant weight) yielded 22.03 per cent. of copper; calculation for the copper salt of prolin being 21.81 per cent. A small amount of crystalline substance, resembling leucin in its properties, was also obtained from this fraction, but not identified.

Fraction 3 yielded but a minute quantity of a sour-tasting, non-crystalline substance, which was too small in amount to identify.

Fraction 4 was examined in the usual way for phenylalanin, but if present, it was in too small quantity to be isolated. Aspartic acid was also sought for, but could not be found.

Fraction 5, the residue left after the fractional distillations, did not yield any phenylalanin, but there was abundant evidence of the presence of some sulphur-containing compound. On hydrolyzing with barium hydrate, and saturating the concentrated, barium-free filtrate with hydrochloric acid gas, there was obtained a precipitate of typical crystals of the hydrochloride of *glutaminic acid.* This was united to the glutaminic acid previously obtained, decolorized with animal charcoal, and recrystallized, pure glutaminic acid hydrochloride to the amount of 1 gram being obtained. This was analyzed, and gave the following figures:

C, 33.15 per cent.; H, 5.61 per cent.; N, 7.70 per cent.

Calculated for glutaminic acid hydrochloride:

C, 32.70 per cent.; H, 5.45 per cent.; N, 7.63 per cent.

The chlorine was removed from the filtrate with lead oxide, and from the filtrate was obtained by condensation and crystallization 0.28 gram of beautiful needles, with an extremely acid taste. A copper salt was formed, which contained 33.2 per cent. of copper, the theory for the copper salt of *aspartic acid* being 32.7 per cent.

DISCUSSION OF RESULTS.

The most interesting feature of the results obtained in this analysis is the quantity and the number of different amino acids that were found free in the liver tissue. The actual quantity of each amino acid that was isolated from 700 grams of liver tissue (which constituted but 72 per cent. of the entire liver) was as follows:

	Gram.
Histidin	0.64
Lysin	1.04
Tyrosin	0.70
Leucin	4.16
Glycocoll	0.20
Alanin	0.30
Prolin	0.35
Glutaminic acid	1.00
Aspartic acid	0.28
Total	8.67

The quantities given above indicate nothing as to the actual amounts of the free amino acids that were present in the liver, as will be appreciated by anyone who has worked with these substances, for our analytic methods are so imperfect that the quantities obtained represent merely minimal figures, and account for only such quantities of each amino acid as I could obtain in sufficient purity for positive identification. How small a part of the total quantity of amino acids that was really present in the liver is represented by the isolated and indentified amino acids, is shown by the fact that the 8.67 grams of amino acids obtained accounts for but about 1.5 grams of nitrogen, out of a total of about 5 grams of non-protein nitrogen that was present in the liver extracts from which the amino acids were obtained. In the entire material used there was but about 64 grams of protein, which would contain about 10 grams of nitrogen; therefore it is noteworthy that nearly one-third of the nitrogen of the liver was in a water-soluble, non-protein form. If we take into consideration the fact that the patient in this case had been sick some six weeks, the question arises: Could so large an amount of readily soluble substances, such as free amino acids, accumulate in the liver and remain in it for any considerable part of this time? If we estimate the protein lost from this liver during the course of the disease, which resulted in a decrease of its fresh weight to 970 grams (the normal being about 1650 grams), and make allowance for the relatively high proportion of water in the diseased liver, we find that it amounts to approximately 210 grams. This would contain approximately 30 grams of nitrogen, so that the soluble, non-protein nitrogen

found in this liver would represent about one-sixth of all the nitrogen that could have been released by the destruction of the quantity of liver tissue that was lost during the six weeks illness. Although it is possible that so large a proportion of the liberated amino acids as this might have been retained in the liver, it hardly seems probable in view of their relatively easy solubility. It may be recalled in this connection that Neuberg and Richter[11] found a total of 2.127 grams of free amino acids in 350 cubic centimeters of blood from a patient with acute yellow atrophy, and estimated that the entire blood must have contained as much tyrosin at this time as could have been formed by hydrolysis of the entire liver. Hence they were obliged to conclude that there must be some other source for the free amino acids than the autolysis of the liver, and they suggested the possibility that they come through the intestinal walls without undergoing the normal synthesis, because of some pathological alteration in this structure. Whatever the source of the amino acids may be, the large quantity of free, soluble, non-protein nitrogen present in the liver in my case would seem to be in favor of the contention of Neuberg and Richter that there must be some source for these substances other than the autolyzing liver itself.

The large number of different amino acids that were found is not more than might be expected in the light of the studies of Fischer and his colleagues. Already leucin, tyrosin, lysin, arginin, and aspartic acid have been found free in the liver or blood of persons dying with acute yellow atrophy or similar conditions. Glycocoll has been found in the urine of phosphorus-poisoned dogs,[12] and Wohlgemuth[13] has also found alanin, glycocoll and arginin in human urine in phosphorus poisoning. So far as I have been able to find in the literature, the finding of prolin (pyrrolidin carbonic acid) and glutaminic acid free in the tissues or fluids of either human or animal material has never been before recorded. There is no evident reason why all the amino acids that have been isolated from proteins by Fischer and others might not be found in dis-

[11] *Deut. med. Woch.*, 1904, xxx, 499.
[12] Aberhalden and Barker, *Zeit. f. physiol. Chem.*, 1904, xlii, 524.
[13] *Zeit. f. physiol. Chem.*, 1905, xliv, 74.

eased tissue in which autolysis has occurred; but the rapid absorption of these substances and their early destruction by desamidizing enzymes cause the amounts present at any one time to be so small as to make their successful isolation by the available methods extremely difficult. In my material, arginin and phenylalanin were sought in vain, and no tryptophan reaction was given by the protein-free extracts. Serin, iso-serin, and cystin were not sought, but a sulphur-containing compound was present among the esters obtained by the Fischer method.

Small quantities of proteoses and peptones seem to have been present, as shown by the biuret reaction of the original aqueous extracts and of the diffusate from the protein solution obtained by cold and hot water extraction of the liver. The quantity of the nitrogen found in the different fractions obtained by the Bigelow and Cook methods, indicated the presence of about one-third gram of proteoses, and one-fourth gram of peptones.

Free xanthin and hypoxanthin were also found in minimal quantities, the latter predominating; the total quantity isolated contained but about 0.1 gram of nitrogen. The failure to obtain guanin and adenin is readily explained, since these substances are known to be soon changed by the guanase and adenase of the liver into xanthin and hypoxanthin.[14]

Wakeman's interesting studies on the changes in composition of the liver of dogs during phosphorus poisoning[15] render a study of the composition of the liver proteins in the human liver during acute yellow atrophy of value. The result of the analysis by Hausmann's method of the insoluble proteins of my specimen of acute yellow atrophy, of two normal livers, and of one liver in delayed chloroform poisoning with extreme necrosis of the liver cells, is shown in the following table:

	Acute atrophy.	Normal (anemic).	Normal (congested)	Chloroform necrosis.
Amid nitrogen.	5.5	3.7	4.8	3.9
Humus "	3.6	3.4	4.9	5.7
Diamino "	26.2	32.8	30.0	30.0
Monamino "	64.8	60.3	60.2	60.3

[14] Jones and Austrian, *Zeit. f. physiol. Chem.*, 1906, xlviii, 110.

[15] *Jour. of Exper. Med.*, 1905, vii, 292.

There seems to be present here, as Wakeman found in his dogs' livers, a decrease in the diamino nitrogen, although this is not so striking as in Wakeman's material. Possibly the slighter decrease observed in the acute yellow atrophy liver depends in part upon an increase in the purins present on account of regenerative cell multiplication, for in the Hausmann method of determining nitrogen distribution the purins are partly precipitated with the diamino compounds. It was impossible to determine the relative proportion of each of the three diamino acids, because of accidental loss after the histidin had been separated. However, the proportion of the nitrogen present in the form of histidin (0.54 gram) to that present as arginin and lysin (0.94 gram) is larger than the normal proportion, and suggests that either the arginin or the lysin, or both, were decreased much below the normal.

Interesting figures as regards sulphur, phosphorus and iron in the insoluble residue of extracted liver tissue were obtained, as follows:

	Acute atrophy.	Normal (anemic).	Normal (congested).	Chloroform necrosis.
Sulphur.	0.82	0.75	0.77	0.79
Phosphorus.*	0.90	0.27	0.21	0.50
Iron.	1.22	0.2	0.4	0.5

* Average of four analyses of each specimen.

In spite of the great loss of parenchymatous tissue in the acute yellow atrophy liver the proportion of sulphur is quite the same as for the normal livers. This is in agreement with the findings of Wohlgemuth[16] in phosphorus poisoning of dogs, the liver tissue of which showed practically no decrease in the proportion of sulphur. The proportion of phosphorus is, however, much increased over the figures for the normal livers, being increased by about four times. This is partly explained by the great proliferative activity of the cells of the stroma and bile ducts in the areas in which regeneration is occurring, resulting in the presence or large numbers of new nuclei rich in nucleic acid; but it does not seem that this proliferation is sufficient to explain a four-fold increase in phosphorus. Probably part of the phosphorus of the cells that

[16] *Biochem. Zeit.*, 1906, i, 161.

have undergone degeneration is present in some insoluble form, for the high figure obtained with the liver in chloroform necrosis shows that necrosis of the cells with disappearance of the majority of stainable nuclei is not associated with a decrease in the insoluble phosphorus. The very considerable amount of iron found in the acute atrophy liver is in agreement with the large amount of blood present in the areas of "red atrophy," where the space formerly occupied by liver cells is filled by dilated capillaries; furthermore, iron-containing pigment is usually found deposited in considerable amounts in the liver in this disease, presumably because of the extensive hemolysis produced by the cholemia.

Gelatigenous substances seem to have been excessively abundant, not only relatively, but to a less extent absolutely. The relative increase is largely the result of the fact that in this disease the parenchyma cells are destroyed while the stroma is not injured, and the absolute increase depends upon the regenerative proliferation of the connective tissue. From 700 grams of liver substance analyzed, 10 grams of gelatin were obtained, which corresponds to 13.8 grams of gelatin in the entire liver, 1.4 per cent. of the entire weight of the fresh substance, and 10.1 per cent. of the weight of the dry, fat-free tissue. A normal human liver was analyzed in the same way, and a total of 9.2 grams of gelatin was found in the entire liver, corresponding to 0.57 per cent. by weight of the entire liver, and 3.2 per cent. of the dry, fat-free substance. Another liver, from an acute case of chloroform necrosis, showed but 1.5 per cent. of the fat-free substance in the form of gelatin.

In common with all other recorded analyses, the proportion of water in the liver was found to be very excessive. This is due chiefly to the filling of the spaces left by the destroyed liver cells with blood, and partly, perhaps, to a considerable degree of intracellular edema (cloudy swelling or hydropic degeneration). But 16.2 per cent. of the entire liver was solids, there being 83.8 per cent. of water. This is seen to agree well with other analyses given in the accompanying table.[17]

The great loss of parenchyma is best shown if we consider that the dry substance of the entire liver weighed but 157 grams,

[17] Modified from Quincke, *Spec. Path. und Ther.*, 1899, xviii, 297.

	Water.	Fat.	Fat-free Dried Substance.
Normal liver.	76.1	3.0	20.9
Acute atrophy (Perls).	87.6	8.7	9.7
" " (Perls).	76.9	7.6	15.5
" " (v. Starck).	80.5	4.2	15.5
" " (Taylor).	85.8	2.0	12.2
" " (Wells).	83.8	2.5	13.7
Phosphorus poisoning (v. Starck).	60.0	29.8	10 0
Fatty degeneration (v. Starck).	64.0	25.0	11 0

whereas a normal liver contains 375 to 425 grams of solids; and furthermore, as the amount of connective tissue was rather increased and the fat not much below normal, the loss of parenchyma must represent at least two-thirds that of the entire liver.

As shown by the above table, the acute yellow atrophy liver does not show an accumulation of fat, having not far from the same amount of fat as the normal liver. The yellow color of the organ, which has commonly been assumed to represent fatty changes, is due to the large amount of bilirubin present; this can be shown by placing the tissue in some oxidizing solution, such as potassium dichromate, when the color of the liver at once becomes green. Even the apparent fat increase observed in many pathological conditions, in which the microscope shows a great amount of intracellular fat in the form of fine granules, while chemical analysis shows no fat increase, is entirely wanting in acute yellow atrophy; for in the two typical specimens that I have had the opportunity to examine with special stains, the amount of fat was strikingly small, only occasional cells being found containing a few granules. Therefore, whatever the cause of acute yellow atrophy may be, it cannot be classed among the steatogenetic poisons. As nearly every other degenerative change in the liver is accompanied by more or less fatty metamorphosis, it is remarkable that in this disease, which causes so great an alteration of the liver, no fatty changes occur. Certainly the cause of acute yellow atrophy must differ in some essential respect from the bacterial toxins, phytotoxins, zootoxins and most of the organic and inorganic hepatic poisons with which we are familiar, since all these cause greater or less fatty changes in the liver. In view of the prominence in this disease of autolytic changes, which are supposed to liberate invisible intra-

cellular fat and to make it microscopically visible, one might expect an abundance of this apparent fatty degeneration, but such is not the case.

The amount of lecithin seems not to have been out of proportion to the amount of fat present in the liver, the amount found corresponding to a total of 4.4 grams in the entire liver, or 0.45 per cent. of the fresh weight. In the liver of a man dying from accident, Balthazard[18] found 1.28 per cent. of lecithin. Orlow[19] found in the liver of infants from 0.29 to 1.34 per cent. of the fresh weight of lecithin. Heffter[20] found about 0.65 per cent. of lecithin in the liver of an executed criminal, and states that he found the proportion of lecithin in the liver of acute yellow atrophy to be decreased, but the cases he describes in support of this statement are probably of some other condition, for the dry weight is given as from 32.1 to 37.8 per cent. of the fresh weight, and of this from 55 to 68 per cent. is ether-soluble material; these figures agree with those of ordinary fatty metamorphosis.

Cholesterin was present to the amount of 3.23 grams in the entire liver, constituting 0.3 per cent. of the entire fresh weight, or 2 per cent. of the dry substance. The normal proportion of cholesterin in the liver is given by Orlow as 0.14 to 0.35 per cent. (fresh weight) in children. I have not been able to find figures on the proportion in the liver of adults, but it would presumably be at least as high, or higher (see note, p. 643).

The finding of small quantities of urobilin, bile salts and creatinin is what would be expected of such a liver, in a person dying with severe icterus. That free lactic acid and volatile fatty acids could not be found does not indicate that they may not have been formed in the living tissue, for they would have been rapidly absorbed and removed.

SUMMARY.

From the liver of a young man who died of typical, "idiopathic" acute yellow atrophy of the liver, after an illness of six weeks, there were isolated and identified the following amino acids: Histidin,

[18] *Compt. rend. Soc. biol.*, 1901, liii, 922.
[19] Abstract in *Biochem. Cent.*, 1907, v, 937.
[20] *Arch. f. exp. Path. u. Pharm.*, 1891, xxviii, 97.

lysin, tyrosin, leucin, glycocoll, alanin, prolin, glutaminic acid, aspartic acid. These were found free in extracts of the liver, and presumably represent products of the autolysis of liver cells, although the amount of soluble non-protein nitrogen present in the extracts was so large as to suggest that there must be some other source for these substances.

Small quantities of free proteoses and peptones, and of xanthin and hypoxanthin, were also found in the extracts.

In the insoluble proteins of the liver the proportion of diamino acids was decreased slightly as compared with normal livers. The proportion of protein phosphorus was increased, probably because of active regenerative proliferation, while the sulphur was normal in amount. Iron was increased because of the large quantity of blood in the liver and the hematogenous pigmentation of the liver cells.

Gelatigenous material was increased both absolutely and relatively, because of the loss of parenchyma and the proliferation of the stroma.

The proportion of water to solids was much increased, there having been a loss of over two-thirds of the entire parenchymatous elements of the liver. The amount of fat, lecithin and cholesterin was not far from that normal for the liver.

In conclusion, it gives me pleasure to express my indebtedness for advice and assistance to Professor Lafayette B. Mendel, in whose laboratory this study was begun. I am also under obligations to Professor A. P. Matthews of the University of Chicago for the use of his laboratory during part of the work.

Note.—Since this article was sent to the publishers I have completed the analysis of the fats and lipoids of three other livers, and the results are shown for comparison with the acute yellow atrophy liver in the following table:

It is apparent that the total amount of lecithin in this acute yellow atrophy liver is very greatly reduced, not only as to the actual amount present, but also in its relation to the other constituents of the liver. It would seem that although the liver has lost even as great a proportion of its fatty constituents as of its proteins, it has suffered an even greater loss in its lecithin. The

	Lecithin.				Cholesterin.			
Specimen Number.*	199	202	203	Ac. Atr.	199	202	203	Ac. Atr.
Per cent. of fresh weight.	1.6	1.4	1.5	0.45	0.26	0.37	0.52	0.3
" " " total dry weight.	6.3	6.25	6.2	2.9	1.0	1.7	1.9	1.8
" " " dry, fat free material.	7.7	8.0	8.1	3.2	1.25	2.1	2.9	2.1
" " " ether-soluble substances.	35.3	28.0	17.3	17.6	5.7	7.4	5.9	11.1
Grams in entire liver.	23.7	22.4	16.0	4.4	3.8	5.95	5.4	3.38

* 199 and 202 are normal human livers, from persons dying suddenly. 203 is the liver of a young man dying from delayed chloroform poisoning, with hepatic necrosis of an extreme type; this case will be the subject of a study which will be reported later.

significance of this observation cannot be estimated until we have more figures on the variations in the lecithins of the liver and other organs in health and disease. It is interesting to note, however, that in the liver showing severe chloroform necrosis, with considerable fatty change, there has also been a decrease in lecithin, although not so marked as in the acute yellow atrophy liver.

The cholesterin has not been so greatly reduced, for this liver shows about the same proportions, and nearly as large a total amount of lecithin, as the controls. The reduction in amount of neutral fats and lecithin causes it to form an unusually large proportion of the ether extract.

ON THE CHEMISTRY AND STAINING PROPERTIES OF CERTAIN DERIVATIVES OF THE METHYLENE BLUE GROUP WHEN COMBINED WITH EOSIN.[1]

By THOMAS M. WILSON.

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The only justification for writing another paper on histological stains so generally employed as eosin and methylene blue, is the fact that something new has been discovered that adapts them for a wider range of usefulness. Working along this line a stain was prepared several months ago that was so satisfactory in some work on the opsonic index[2] done by the writer that he considered it worth describing at some length.

CHEMISTRY OF EOSIN.

In 1871, Baeyer[3] found that by treating at 195° C. phthalic anhydride with resorcinol, a yellow mass was formed, slightly soluble in alcohol, which he called fluorescein, $C_{20}H_{12}O_5$, the mother substance of eosin. It is insoluble in water, ether and benzol. Its readily formed alkaline solutions show beautiful fluorescence from which can be got bright yellow star crystals; from alcohol the crystals are small dark brown. It is precipitated by acids, appearing as a brick-red powder.

The free acid eosin, tetrabrom fluorescein, $C_{20}H_8Br_4O_5$, is strongly dibasic and was first described by Baeyer[4] in 1876, although two years prior it was made commercially. The dye workers prepared it by taking one molecule of fluorescein and four molecules of acetic acid, adding thereto twenty per cent. solution of bromine. The potassium salt was next formed and the eosin precipitated by dilute sulphuric acid from which the stain was extracted by ether. The alcoholic solution on slow evaporation yields beautiful yellowish-red crystals with a formula $C_{20}H_8Br_4O_5(C_2H_5OH)$. It is precipitated from the watery solution by mineral acids, as yellowish-red amorphous eosin, the latter being more soluble in alcohol than the crystalline form. In the presence of the slightest trace of alkalies, a red-yellow color, and a beautiful yellow-green fluorescence is produced. It is almost insoluble in chloroform and benzol. On reduction with zinc

[1] Received for publication July 12, 1907.

[2] Wilson, *Amer. Jour. of Physiol.*, 1907, xix, 445.

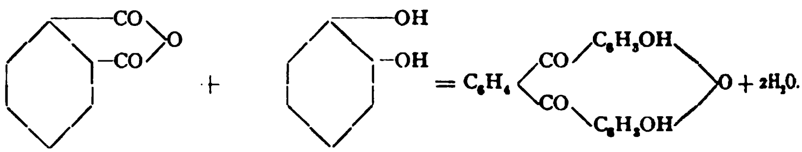
[3] Baeyer, *Berichte Deut. Chem. Gesel.*, 1871, iv, 558.

[4] Baeyer, *Liebig's Annal.*, 1876, clxxxiii, 38.

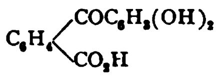
dust and alkalies it becomes colorless. Salts are readily formed by treating it with the corresponding base. The alkali salts are soluble in water, the earthy salts slightly so, while those of the heavy metals are almost insoluble. Silver eosinate, however, is somewhat soluble in water and alcohol. Water-soluble eosin is the potassium or sodium salt. The formula of the former is $C_{20}H_6Br_4O_5K_2 5H_2O$, and its molecular weight 824. It is soluble in two parts of water, and slightly soluble in absolute alcohol. Its tri-clinic crystals are red by transmitted light with a yellow-green lustre. The dilute solution is red-yellow, with strong green fluorescence. Its absorption spectrum shows a broad black band in the green.

Our knowledge of the constitution of eosin depends primarily on the manner of linking one molecule of phtalic anhydride and two molecules of resorcinol, so as to form one molecule of fluorescein and two molecules of water. E. Fischer[5] wrongly claimed that it was done as follows:

Phtalic Anhydride. Resorcinol. Fluorescein. Water.



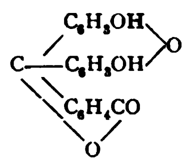
Baeyer[6] found that by heating mono-resorcin phtalein



that fluorescein and phtalic acid were formed according to the equation

$$2C_{14}H_{10}O_5 = C_{20}H_{12}O_5 + C_8H_6O_4 + H_2O,$$

and concluded that it was a derivative of phenolphtalein anhydride, representing it semi-graphically thus:

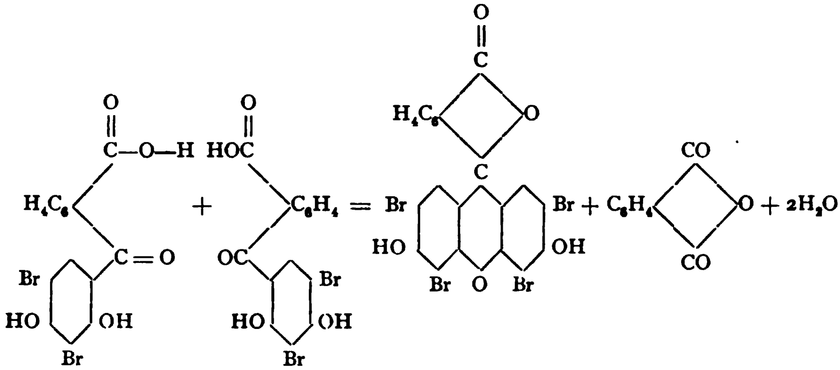


Keller[7] fixed one hydroxyl in a resorcinol radicle in the para-position with regard to its attachment to the phtalic radicle.

R. Meyer[8] obtained 3, 5, dibrom; 2, 4, dioxy-benzoyl-benzoic acid, and some phtalic anhydride by heating eosin above its melting point, and concluded that eosin was formed as graphically shown below.

[5] Fischer, *Berichte Deut. Chem. Gesel.*, 1874, vii, 1211.
[6] Baeyer, *Liebig's Annal.*, 1876, clxxxiv, 24.
[7] Keller, *Berichte Deut. Chem. Gesel.*, 1895, xxviii, 321.
[8] Meyer, R., *Berichte Deut. Chem. Gesel.*, 1896, xxix, 2624.

3, 5 Dibrom, 2, 4 Dioxy-benzoyl-benzoic Acid. Eosin. Phtalic Anhydride Water.



According to this lactone theory, eosin is tetrabrom-dioxy-fluorane. It possesses two heterocyclic rings, one five-membered, the other having six atoms. In the former is found two acid ketonic chromophores, $= C = O$. One of them is, however, replaced by an oxygen atom, $= O$, and in the six-membered ring there is another similarly substituted ketone. Linked on, as side chains, are two acid auxochromes, $O — H$, which both intensify the color and aid in the electrical dissociation. The addition of bromine atoms increases the color toward the blue end of the spectrum.

CHEMISTRY OF CERTAIN METHYLENE BLUE DERIVATIVES.

Methylene blue was first prepared in 1876 by Caro,[9] and a few years later Bernthsen[10] published two articles on the chemistry of the whole methylene blue group. Bernthsen's is still our best single source of information on this subject.

Methylene blue is prepared by oxidizing dimethyl-phenylene diamine with ferric chloride in the presence of hydrogen sulphide. Its formula is $C_{16}H_{18}N_3SCl + 3H_2O$ and its molecular weight 373. At 100° C. it possesses three molecules of water, but loses these at 150° C. It crystallizes, by means of sodium chloride and hydrochloric acid, in small glittering microscopic leaflets, which have either a copper or bronze-colored lustre which, on cleavage, shows a cantharides gloss. It is soluble in water and alcohol. Its cold blue aqueous solution is not destroyed by strong hydrochloric acid, weak nitric acid or by alkalies. It is not destroyed by boiling its aqueous solutions. It forms a base in the cold with fresh silver oxide. By long boiling with weak alkalies or silver oxide, it yields methylene violet, methylene azure, and their leuco-bases, besides other lower derivatives, such as dimethyl-amine. Its spectrum in dilute solution gives a dark band between B and C, and a dim one to the left of D, with the maxima at λ 670 and λ 610, respectively.[11]

[9] Caro, *Berichte. Deut. Chem. Gesel.*, 1876, xi, 360.
[10] Bernthsen, *Liebig's Annal.*, 1885, ccxxx, 137; also 1889, ccli, 1.
[11] λ equals a thousandth part of a μ.

Methylene azure,[12] $C_{16}H_{18}N_3SO_2$, is an oxidation product formed by boiling weak alkalies or silver oxide with methylene blue. Its direct separation in pure form from methylene violet has not been accomplished, but by converting these bodies into their leuco-compounds the azure can, by alkalies, be separated from the leuco-methylene violet. After this process, the leuco-azure can, by oxidation, be readily reconverted into the methylene azure. It is soluble in ether, with raspberry color; in water, with blue color; while in alcoholic and chloroform solution it is violet red, and in benzolic and xylolic, pure red. The leuco-compound obtained by alkalies and stannous chloride is insoluble in alkalies.

Methylene violet, $C_{14}H_{12}N_2SO$, is also formed in the boiling of methylene blue with an excess of silver oxide. Its crystals separate out, and can be purified by dissolving them in hot alcohol, to which it gives a violet color and red-brown fluorescence. It is slightly soluble in ether, cold or hot water, and more soluble in chloroform and acetone. It forms long black greenish glittering needles. The hydrochloride of the violet is obtained by crystallizing the base with dilute hydrochloric acid, when beautiful centimeter-long crystals appear.

Leuco-methylene blue present in the precipitate formed by boiling silver oxide with methylene blue is soluble in ether, cold alcohol, and slightly in water. It crystallizes in broad atlas glittering needles and oxidizes in the moist condition very easily, forming methylene blue. It is insoluble in strong potassium hydrate. Like other leuco-derivatives of methylene blue, it is colorless, due to the loss of its double nitrogen bond, and the introduction, therefore, of hydrogen.

Methylene red is always present in the mother liquid from which methylene blue is prepared. It is soluble in water, and alcohol, giving thereto a violet-purple color. It is not soluble in ether. The formula for the chloride was given as $C_{16}H_{18}N_4S_4 2(HCl)$, which later research has shown should be halved.

A last compound of interest is the free base of methylene blue, $C_{16}H_{18}N_3SOH$, which is obtained from the chloride by treating it several days with luke-warm, freshly prepared silver oxide. On evaporating this solution over sulphuric acid, a green amorphous mass with metallic lustre remains. It is insoluble in ether, but very soluble in water and alcohol.

The Structure of Methylene Blue.

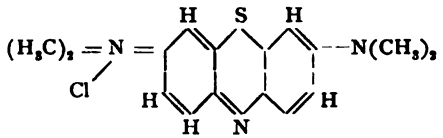
The different methods of preparing thionin, or Lauth's violet,

$$H-N\begin{matrix}\diagup C_6H_3NH_2 \diagdown \\ \diagdown C_6H_3NH_2 \diagup\end{matrix}S,$$

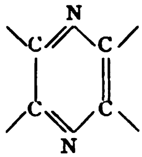
from (*a*) p-phenylene diamine, (*b*) thio-diphenyl amine, (*c*) p-diamido-diphenylamine, show that its real structural name should be diamido-diphenthiazine, and since it is the mother substance of methylene blue, it furnishes the starting point for determining the constitution of the latter body.

Methylene blue, according to the quinone theory, is the chloride of tetramethyl-diamido-diphenthiazine.

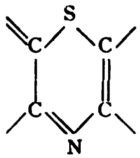
[12] Michaelis, *Chem. Cent.*, 1901, ii, 708.



The termination "azine," also called azinium, indicates the substitution of one or more methin groups by nitrogen atoms in a six-membered mono-hetero-



atomic ring. For example, the radicle would be a p-diazine. If, now, one of the nitrogen atoms in it were replaced by a sulphur atom, the group would then be called p-thiazine.



It would consist of one sulphur, one nitrogen and four carbon atoms. It would still be a stable body, since the replacement of methin groups by nitrogen, or the second replacement of nitrogen by sulphur, affects the stability of a six-membered ring very slightly, as shown by the high melting and boiling points of many compounds in which this ring occurs. The strong basic chromophore indamin $-N=$ occurring as part of the thiazine ring has two of its bonds united to one benzene ring, while the third is linked to the other benzene. The presence of the sulphur atom in the thiazine group might be regarded as a substituted ketonic acid chromophore, $=C=O$ becoming $=S$, lessening, however, the strong basicity of the indamin, but still leaving p-thiazine decidedly chromogenic. This group linked with the two benzene rings forms dipenthiazine.

A glance at the graphic formula of methylene blue will show that there is a substituted hydrogen atom in each of the two benzene nuclei which occupies the para- position with regard to the nitrogen in the thiazine group. If, then, one of these hydrogens is replaced by a dimethylamine and the other hydrogen by a double-bonded nitrogen, carrying two methyl groups and one chlorine atom, the resulting compound will be methylene blue. In Bernthsen's theory, the nitrogen in the heterocyclic ring is united to the nitrogen carrying the chlorine atom. The strongly basic auxochrome, NH_2, attached to one benzene ring does not influence the dissociation of chromogen, as do the other salt formers. It, however, intensifies the color of the chromophores and gives the dye a basic character. It never dissociates electrically, but it influences other nitrogen atoms which may be present and gives them power to unite with the tissues. The

center of each spectrum band is moved λ 15 for each methyl group introduced. The substitution of the two methyl groups in the amidogen side chain renders the stain darker and slightly less basic. Why these phenomena occur is still unknown. In the other benzene ring there is present the group $=N(CH_3)_2Cl$ holding on to its benzene ring by a double bond. It may, therefore, be considered as a substitution for the oxygen in the acid chromophore, $=C=O$, in which the oxygen atom is replaced by two of the bonds of a pentad nitrogen. This replacement not only annuls the acid power of the chromophore but renders it decidedly basic. Moreover, the presence of this pentad nitrogen renders the whole dye a chloride of an ammonium base which dissociates into the colorless anion Cl and the remainder, the colored kation. Thus the presence of the three basic chromophores more than counterbalances the single acid one and so the compound as a whole is basic.

HISTORY OF METHYLENE BLUE AND EOSIN STAINING.

Although eosin was first made in 1874, and methylene blue two years later, it was not till 1891 that Romanowsky[13] first employed a combination of the two for histological purposes. In staining for Plasmodium malariæ, he sought to obtain an aqueous solution of a neutral compound of the two ingredients. He, therefore, took one part of a saturated solution of methylene blue and two parts of one per cent. water-soluble eosin. His sections fixed by heating for one hour at 105° C. sometimes required several hours to bring about the desired staining effect. His successful results with human blood which did not always occur, are given in Table I.

Unna[14] founded the ingenious method of special contrast staining of certain tissue cells by using polychrome methylene blue. He obtained metachromatic staining of Bacillus tuberculosis, *mast,* and plasma cells by alkalinizing ordinary methylene blue, and thus obtaining some methylene violet and methylene azure, which latter substance he wrongly called methylene red.

Mathews[15] was the first to bring out the fact that staining with methylene blue, as with any other basic dye, depends upon the alkaline reaction of the tissues.

Ziemann,[16] who made use of the same stain as Romanowsky, observed that the precipitate formed upon the union of eosin with methylene blue was essential for the success of the stain, and was soluble in an excess of either constituent. By adding borax, potassium hydrate or sodium carbonate, he could always get a body capable of staining plasmodia. This body he supposed to be a compound of eosin. He employed it in staining flagella, fungi and various bacteria. It gave the same results as a good Romanowsky stain, and was more certain, for reasons to be shown later.

Laurent[17] united the dyes according to their valencies. Methylene blue being

[13] Romanowsky, *St. Petersburg. med. Woch.*, 1891, viii, 297.

[14] Unna, *Zeit. f. wissensch. Mikros.*, 1892, viii, 475.

[15] Mathews, A. P., *Amer. Jour. of Physiol.*, 1898, i, 449.

[16] Ziemann, *Cent. f. Bakt.*, 1898, xxiv, 945.

[17] Laurent, *Cent. f. allg. Path. u. Anat.*, 1898, ix, 86.

monobasic requires twice its molecular weight to neutralize one volume of the dibasic acid, eosin. After thus forming the precipitate, it was kept in well-stoppered bottles, and before using, was shaken and a portion diluted four times. It was then boiled for half an hour, thus dissociating the neutral salt. The colored ions remain in this dissociated condition for five or six hours. The time required for staining with the cold liquid was from thirty minutes to five or six hours. The specimens were differentiated in absolute alcohol.

Rosin[18] points out that when methylene azure solution is evaporated it forms a red-violet body. This is the essential ingredient in Unna's polychromatic stain, and forms in long-standing methylene blue. The variability[19] of the staining reaction of methylene blue and eosin led to his finding five different bodies soluble in alcohol and water, but differently soluble in chloroform. This latter fact enabled them to be separated. One body he designated methylene blue eosin. Its blue violet needle-shape crystals[20] have a metallic lustre and decompose on exposure, becoming dark brown. They are insoluble in cold water, but the washings will continue for days to show a bright rose tinge with green fluorescence. In hot water they are slightly soluble, with strong fluorescence. The body is difficultly soluble in alcohol, with blue violet-green fluorescence. Mineral acids change this color, which fails to return on neutralization. With organic acids, the color of alcoholic solutions varies from pure blue to blue green. Alkalies change the solution to red, and violet blue returns on neutralization. It is slightly soluble in chloroform, with red violet color, and no fluorescence; but absolutely insoluble in ether. It stains celloidin, mucin, nuclei, and Nissl granules blue, while protoplasm of nerve cells, proteids, fibrin, and even glass become rose-red. Cytoplasm strikes a red tint, and neutral granules become violet. He claims that this stain, like all others formed by the union of basic and acid dyes, differentiates the tissues into their component parts, staining the nuclei blue and the cytoplasm red. He also claims that methylene azure and methylene violet are two other bodies formed in uniting methylene blue and eosin. This statement has been indirectly dealt with in discussing the work of Bernthsen, and will be referred to later. Methylene orange, a fourth compound, is very soluble in ether and appears to be nothing but tetrabrom fluorescein derived from the water-soluble eosin. The remaining substance is a black dye, insoluble in ether, but soluble in chloroform.

Zettnow[21] shows how to treat the methylene blues manufactured by the different German firms, with alkalies to get the best results in staining bacteria and blood. In differentiation, he used 0.2 per cent. eosin, and 0.1 per cent. methylene blue.

Nocht[22] showed that neither polychrome methylene blue nor eosinate of methylene blue taken singly, but a combination of the two was essential for the staining of malarial parasites. Hence it was that Unna's polychrome added to Romanowsky's mixture increased the intensity of that stain. Nocht's directions

[18] Rosin, *Deut. med. Woch.*, 1898, xxiv, 616.
[19] *Idem.*, *Cent. f. Physiol.*, 1899, xiii, 561.
[20] *Idem.*, *Berl. klin. Woch.*, 1899, xxxvi, 251.
[21] Zettnow, *Zeit. f. wissensch. Mikros.*, 1899, xvi, 254.
[22] Nocht, *Cent. f. Bakt.*, 1898, xxiv, 839.

required 1 c.c. of neutralized polychrome methylene blue (litmus paper as a indicator) and 1 c.c. of water, then ordinary concentrated solution of methyle blue till the polychrome color had changed to a clear blue. This mixture is nc added to 0.1 per cent. water-soluble eosin. The specimens took up this stain from three to twenty-four hours, and were differentiated by very dilute acet acid. In his second paper,[33] Nocht modified the stain by treating at 50° to 60° ordinary one per cent. methylene blue with 0.5 per cent. sodium carbonate, co ing and adding it to one per cent. eosin, until the resulting color shows no re A slight excess of either does not affect the stain. Nocht avoids committi himself as to the real nature of the ingredient essential for staining malar parasites by calling it "*Rot aus Methylenblau.*" He claims it is neither methyle red nor methylene violet. A water solution of fresh methylene blue gives chloroform a blue tint, while a polychrome solution gives to it a red color. study of Bernthsen would have thrown light on this color-producing materi

Laveran,[34] who discovered the parasite of malaria in 1881, worked on th compound stain. He employed the free base of methylene blue, called by hi *Bleu Borrel,* after Dr. Borrel, who prepared it by adding fresh silver oxide any methylene blue, and letting it stand for several days. 1 c.c. of this base w added to 5 c.c. of 0.1 per cent. eosin and 4 c.c. of distilled water. This stain the nuclei of the hæmatozoa violet and their protoplasm blue.

Jenner[35] simplified this stain greatly. He united equal volumes of 1.5 pe cent. eosin and one per cent. methylene blue and collected the precipitate, whic was dried at 55° C., and dissolved in pure methyl alcohol. The air-dried smea of blood was flooded with the stain for one to three minutes which was the poured off; the film was rinsed in distilled water, dried in the air, and mounte in balsam. He claims that good results can be obtained by making separate alcoholic solutions of methylene blue, and eosin, and then mixing them.

Leishman[36] made a solution of 0.5 per cent. of sodium carbonate in one pe cent. methylene blue, heated it at 65° C. for twelve hours, and let it stand fo ten days. To this he added an equal volume of 0.1 per cent. eosin and dissolve the precipitate in pure methyl alcohol. He applied the stain in the same manne as Jenner, leaving it undiluted on a blood specimen one and one half minute then diluting it with double the volume of distilled water and allowing it to ac for from five to ten minutes.

Wright[37] steams at 100° C. one per cent. sodium bicarbonate for one hou and after cooling adds 0.1 per cent. eosin till a purple color is formed with metallic scum. He states the proportions as two of methylene blue solutio to one of eosin. The collected precipitate is dried and dissolved in pure methy alcohol to make an 0.5 per cent. solution. He applies it by nearly flooding th slide containing the air-dried specimen for one minute with the stain. Wate drop by drop, is next applied until a scum forms, but not enough to make th fluid transparent. In this condition it remains from two to three minutes. He

[33] Nocht, *Cent. f. Bakt.*, 1899, xxv, 764.

[34] Laveran, *Compt. rend. de la Soc. de Biol.*, 1899, li, 249.

[35] Jenner, *Lancet*, 1899, i, 370.

[36] Leishman, *Brit. Med. Jour.*, 1901, i, 635; 1901, ii, 757.

[37] Mallory and Wright, Pathological Technique, 1904, 3d edition, p. 370.

TABLE I.

STAINING EFFECTS.

	Romanowsky.[28]	Nocht.	Jenner.	Leishman.	Wright.	Eosinate of Thionin.
Red corpuscles.	Rose red.	Rose to brown red.	Terra cotta.	Pale pink or greenish tint.	Orange or pink.	Pale blue.
Platelets.			Mauve.	Red.	Dark lilac.	
Nucleated reds.	Deep violet.			Ruby red (fine red granules).		
Lymphocytes.	Blue.				Purplish blue nuclei; robin egg blue cytoplasm.	Purplish blue.
Large monon. leuc.						Lavender.
Nuclei of polym. leuc.		Carmin violet.	Pink.			Purple.
Granules of polym. leuc.	Bright violet.				Red lilac.	Pale red.
Granules of eosinophiles.			Red.	Pale pink.	Eosin.	Reddish.
Granules of basophiles.			Violet.	Greenish purple or black.	Purplish.	Purple.
Nuclei of plasmodia.	Carmin violet or bright red.	Bright red.		Ruby red.	Lilac red to black.	
Protoplasm of plasmodia.	(Granules black) clear blue.	Pure blue.			Blue.	
Protoplasm of white corpuscles.	Blue.	Blue or eosin.				Pale blue.
Bacteria.			Blue.	Blue.		Dark purple.

[28] Jackson, Tropical Medicine, 1907, p. 320.

differentiates by means of water, examining microscopically until the red corpuscles become pinkish. When the proper color develops, the specimen is quickly dried between layers of filter paper and mounted in Canada balsam.

The staining results of the different investigators are given in Table I., and along with them, those brought out by using eosinate of thionin.

This survey of the literature and examination of the chemistry shows that eosin and certain derivatives of the methylene blue group are essential to obtain certain effects in the staining of blood. It was first pointed out by Nocht that eosinate of methylene blue stains the basic cytoplasm and the acid chromoplasm, and the presence of "*Rot aus Methylenblau*" produces the characteristic malarial parasite staining. Nocht, however, failed to state what that specific staining body really was chemically. By Rosin and others it was assumed to be methylene azure, although this was not demonstrated. As to the staining functions of other derivatives of methylene blue which are invariably present, nothing is recorded.

For the purpose of throwing light on these problems, and, if possible, making the preparation of these stains less empirical, an attempt was made to isolate these derivatives and study their staining reactions for normal and abnormal elements in the blood, and also, to ascertain what bodies are formed by the action of the silver oxide or dilute alkalies, and finally, by using the facts acquired, to treat a specimen of methylene blue so as to obtain a known mixture of its chief derivatives, and then to combine them under proper conditions with eosin.

THE ISOLATION OF CERTAIN METHYLENE BLUE DERIVATIVES.

Preparation of Methylene Violet.

Methylene violet was prepared by boiling 0.5 per cent. methylene blue with 0.5 per cent., silver oxide for about six hours. A drop placed on the slide showed microscopically, besides the silver debris, a purple flocculent precipitate, soluble in ether, and crystals in the form of sea urchins, whose rays were dark brown by transmitted light, and when dried, dark green by reflected light. These latter forms were insoluble in both cold water and ether, but soluble in hot alcohol. The boiled methylene blue was extracted with ether

and the filtered residue first washed with cold water, and then treated with hot alcohol. The resulting solution gave on standing in an atmosphere of hydrogen almost pure crystals of methylene violet. Suspended in dilute hydrochloric acid, one sees the crystals disappear, surrounded by a purplish halo. The resulting small star crystals of the hydrochloride were found insoluble in 0.5 per cent. hydrochloric acid, soluble in cold 95 per cent., insoluble in 10 per cent. alcohol and insoluble in distilled water, dilute alkalies and ether. The purplish alcoholic solution gives with sulphuric acid, a port wine color. Chloroform dissolves the crystals, with red brown fluorescence. Tar soap gave with the alcoholic solution a pretty green color. The methylene violet prepared as described above was soluble in xylol, benzol, and toluol, with red color. Potassium bichromate gave a dark carmin brown precipitate, made up of star crystals about seven μ in diameter. Zinc chloride, and hydrochloric acid gave violet fluffy precipitate. A solution of the methylene violet in methylated spirits stained the nuclei purple, and the red corpuscles greenish blue. Methylene violet will not unite with eosin in alkali or in neutral solutions. The hydrochloride, however, of the methylene blue readily forms a purplish compound with eosin which stains blood smears faintly.

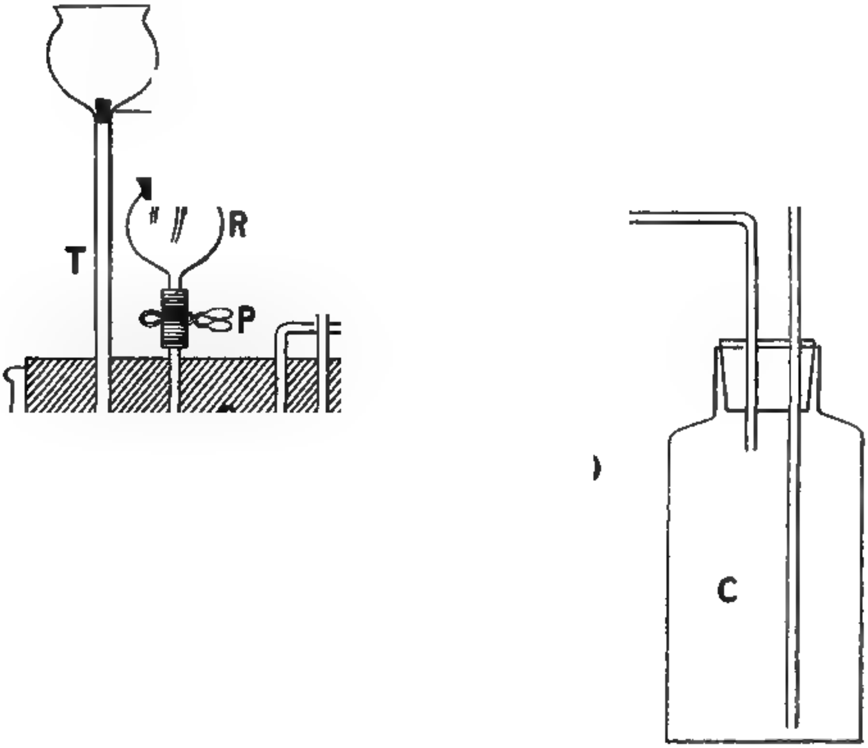
Preparation of Methylene Azure.

The isolation of methylene azure was more difficult. Ether as its solvent was discarded, because methylene violet was also found in the extract. Partial precipitation of a chloroform extract also proved unsatisfactory, since the high concentration at which separation occurred was so great that, although the filtration was performed in an atmosphere of chloroform, no reliance could be placed on the purity of the product. It was found, however, that leuco-methylene blue and leuco-methylene azure are insoluble in sodium hydrate, whereas leuco-methylene violet is soluble. If, then, the boiling with silver oxide were continued till all the methylene blue had disappeared, the other two bodies could be separated. For the purpose of ascertaining when this disappearance occurred, 1 per cent. methylene blue, 1.5 per cent. silver oxide, and 0.5

per cent. sodium bicarbonate were boiled together. Extracts from the specimens slightly acidulated with hydrochloric acid were made with chloroform at intervals of ten minutes. This process removed the methylene azure and the violet, from the other bodies insoluble in chloroform. The filtrate from the residue was treated with hydrochloric acid. The persistent blue color, even when extremely diluted and treated with ammonia water proved the presence of methylene blue. In this way it was found that in forty-five minutes all the methylene blue had disappeared from the material. A similar specimen, boiled forty-five minutes, was filtered so as to remove the debris, and most of the methylene violet and the filtrate reduced by means of a hot strongly alkaline stannous chloride solution. To it was added half its volume of alcohol, and the resulting leuco-methylene azure was allowed to crystallize. The product was filtered and washed in an atmosphere of hydrogen. The leuco-base was oxidized into the hydrochloride and then studied. It was found to stain the red corpuscles green, the lymphocytes blue, and the nuclei of the polymorphonuclear leucocytes deep purple. These staining effects were found to be identical with those from the same methylene blue mixture when boiled thirty minutes with hydrochloric acid and then neutralized.

Several experiments led to the inference that methylene azure does not unite with eosin when the latter is added to a methylene blue mixture containing azure. Ether is a solvent for methylene azure. When an extract of the latter is evaporated, the residue is very slightly soluble in water or dilute alkalies, and no precipitate is detected microscopically when eosin is added to the saturated blue aqueous solution. If, however, the extract is evaporated in an atmosphere of hydrogen, or if some acid is first added, then a fine pale purplish precipitate is visible under medium magnification. Care in the latter case, however, must be exercised to avoid so much acid as to precipitate the yellowish red tetrabrom-fluorescein from the water-soluble eosin. Proof of the correctness of the inference was further secured by observing that as much matter soluble in ether was obtained from the mixture treated with eosin as from that untreated with it.

The apparatus used in the filtration of leuco-methylene azure and of other bodies to be dealt with later is shown in the diagram which follows.



The small bottle, *B*, with funnel and filter was placed in the large bottle, *A*, containing some water and zinc. Through its large rubber stopper, *S*, pass four tubes fitting snugly to prevent leakage. The tube, *T*, dipping under the water in *A* is closed by a small rubber cork, *K*, which is only removed when acid is being

added for the generation of hydrogen. *R* is a thistle tube with rubber connection and attached clamp to permit the entrance of the fluid to be filtered. On opening the clamp at *O* the pressure of the hydrogen forces the filtrate into the bottle *D*. When the clamp on *H* is open, hydrogen passes into the corked bottle *C* containing the material for filtration. On closing the outlets from *A* the pressure drives the solution through the open tube, *M*, into the receptacle of the thistle tube, *R*. As soon as the latter is filled, *S* is clamped, *O* is opened, and after the pressure has gone down, the pinch cock, *P*, is opened, and the material passes on to the filter paper.

The Examination of Thionin, Thionolin and Thionol.

These three bodies should receive a passing notice, since their presence was strongly suspected in the boiled methylene blue solution along with the two bodies already identified. Thionol was prepared from thionin by boiling it with dilute sodium hydrate, and the product, supposedly thionolin, formed by boiling thionin for one hour with 500 times its volume of water. The three substances were examined chemically, and the results are recorded in Table II, along with those of the two other products derived from methylene blue.

On Certain Bodies Obtained from Methylene Blue when Boiled with Dilute Alkalies.

I next sought a rapid simple method of identifying the methylene violet and methylene azure already isolated, and estimating approximately the quantity of each produced by the ordinary treatment of methylene blue with dilute sodium bicarbonate.

The spectrum of a dilute solution in alcohol obtained from the dry ether extract gave an absorption band, reaching from 665λ to 541λ, and darkest at 628λ. It occurred towards the red end of the spectrum and terminated abruptly, while towards the portion of the spectrum where the rays were least refrangible, the band was dim and gradually faded away. An alcoholic solution from a second chloroform extract gave a band from 651λ to 550λ, darkest at 594λ. This analysis indicates that ether and chloroform extract different bodies, hence these solvents were considered best adapted for my purpose. Ether dissolves chiefly methylene azure, while chloroform extracts both the azure and the violet, and

TABLE II.

Reagent.	Methylene Blue Chloride.	Methylene Violet.	Methylene Azure.	Thionin.	Thionolin.	Thionol.
Crystals.	Long, thin, bluish needles. Small glittering leaflets. Blue violet.	Rhomboids deep brown, sea urchins, sheaves, needles. May be purplish brown with greenish color on reflected light.	Dirty brown. Occasionally needles or hair-like forms.	Forms yellow precipitate. Microscopic needles. Sometimes black crystalline powder. Weak green shining crystals.	Metallic green powder.	Brown red, fine, dirty precipitate. Metallic green.
Cold water.	Blue. Soluble.	Insoluble.	Nearly insoluble.	Almost insoluble.	Slightly soluble, with red violet color in all dilutions.	Insoluble.
Hot water.	Blue. Soluble.	Soluble.	Purplish blue. No. fluorescence.	Soluble.	Violet color.	Slightly soluble. Reddish color.
Alcohol.	Blue. Soluble.	Soluble in hot 10 per cent. alcohol	Bluish red. Very faint fluorescence, purple on reflected light.	Difficultly soluble. Red brown fluorescence.	Violet. Soluble.	Deep red with violet-red fluorescence.
Ether.	Insoluble.	Insoluble as shown by crystals.	Fiery red. Very slight fluorescence.	Insoluble.	Almost insoluble. Slight tinge of red.	Very slightly soluble. Yellow red. Slight fluorescence.
Chloroform.	Insoluble. Slight blue tinge.	Very soluble.	Purple color. Slight fluorescence.	Insoluble.	Difficultly soluble.	Red yellow. Slightly soluble.
Carbon bisulphate	Insoluble.	Soluble.		Slight trace. Insoluble.	Insoluble.	Insoluble.
Toluol.	Insoluble.	Red color.	Red color.	Insoluble.	Insoluble.	Insoluble.
Ligron.	Insoluble.	Soluble.		Insoluble.	Insoluble.	Insoluble.
Benzol.	Insoluble.		Red solution.	Insoluble.	Difficultly soluble.	Red. Slightly soluble.
Hydrochloric acid	Blue.	Soluble, but the crystals are insoluble in dilute HCl. Sea urchin forms.	No precipitate. Greenish colored solution.	Slight red color. Forms dark purple precipitate with $HgCl_2$ insol. in cold; sol. in hot.	Deep red, strong blue, then violet.	Fine, green, glittering, straight or needles.

TABLE II.—*Continued.*

Reagent.	Methylene Blue Chloride.	Methylene Violet.	Methylene Azure.	Thionin.	Thionlin.	Thionol.
Potassium bichromate.	Needles.	Forms fine pale carmin-brown precipitate. Star crystals, of R. C. size	Purplish precipitate.	Brown precipitate.	Deep red precipitate.	Slight precipitate.
Zylol.	Insoluble.	Red.	Red.	Insoluble.	Insoluble.	Insoluble.
Sulphuric acid.	Green.	Blue. Soluble.	Green in solution.	Green color.	Decomposes by heating. Forms Thionol.	Blue in strong acid. Violet reflex. In dilute acid, violet-red to red.
Zinc, chloride and sodium hydrate.	Purple.		Deep red brown.	No precipitate.		
Dilute alkalies	Blue.	Insoluble.	Slightly red.	Deep red color, then precipitate.	Deep red.	Violet-red.
Eosin.	Dark blue precipitate.	No precipitate.	Precipitate pale purple; slight.	Purple precipitate.	Fine purple star, burr formed crystals.	Deep purple-red precipitate.
Potassium iodide.	Bluish precipitate.		Light brown, fluffy precipitate.	Dark purple.	Light violet.	Brown precipitate.
Aniline.	Green.			Green, soluble which become yellow.	Greenish color.	Red-brown color. Soluble.
Alcohol and sulphuric acid.	Green. Soluble.	Yellowish.	Yellow. Turns brown.	Green. Soluble.	Insoluble.	Eosin color. Soluble, afterwards becoming brown.
Sodium hydrate. Strong.	Blue.	Deep red.	Deep red.	Forms red-brown precipitate.	Forms yellow precipitate of thionol.	Violet color.

methylene blue remains insoluble in both. An ether extract, therefore, contains chiefly the azure, and the residue will have lost its red color, while the methylene violet will be obtained in the second extraction by chloroform. It was therefore proposed to treat different samples successively with ether and chloroform, and estimate by colorimetric methods the amount of the solutes present. A quantity of one per cent. methylene blue, containing 0.5 per cent. sodium bicarbonate, was boiled in a graduated beaker and from it at definite intervals, ten cubic centimeters were transferred into labelled bottles, care being taken to add distilled water to make up for evaporation between the intervals of extraction. From the five minute specimen bottle, four cubic centimeters were transferred to a clean dry test tube to which was added 2.5 times the amount of ether. The mixture was then shaken gently (not vigorously), so as to avoid, as far as possible, carbon dioxide uniting with the unstable azure. Of this extract, eight cubic centimeters, freed from water and from any methylene blue, were transferred to another test tube, which was afterwards filled with coal gas and tightly corked. This pale red solution was used as a color unit. The other tubes were extracted with ether and records kept of the number of dilutions necessary to yield the same tint as the unit. The result, divided by 2.5 gave the quantity of the azure in the specimen as compared with the original. The methylene violet determinations were similar, but on account of the more intense color, much smaller quantities of the specimens were employed for extraction. The standard color unit in this case was obtained by diluting 0.5 cubic centimeter of the ten-minute specimen, twenty times, that is, by adding ten cubic centimeters of chloroform. The findings of another similar experiment wherein 0.2 per cent. sodium bicarbonate was employed instead of the 0.5 per cent., where the same color units of the former series of experiments were retained as units in this shorter series, showed a much smaller increase of material soluble in ether and chloroform, during the sixty minutes' boiling.

The results of both series given in Table III show that the amount of material in the ether and chloroform extracts depends

on the duration of the boiling and the strength of the alkali; and further that the substances soluble in each solvent do not increase in the same proportion.

TABLE III.

TIME IN MINUTES.

	0 m.	5 m.	10 m.	20 m.	30 m.	40 m.	50 m.	60 m.	75 m.	105 m.
One per cent. MbCl boiled with 0.5 per cent. $NaHCO_3$ extracted with ether.	0	1	5	40	80	110	180	194	660	480
The same extracted with chloroform.	0		1	3.5	5	6	6	7.2	14	18.6
One per cent. MbCl boiled with 0.2 per cent. $NaHCO_3$ extracted with ether.	0	1	2	3	3.6			6		
The same extracted with chloroform.	0		0.2	0.4	0.5			2.4		

It was noticed microscopically that at least two distinct forms of colored crystals resulted from methylene blue when boiled with dilute alkalies, but since the literature mentions only one red colored crystalline form, viz., methylene violet, as being thus formed, it was assumed that another compound was present.

Evidence that at Least Four Bodies are Formed.

Various reagents insoluble in water, suitable for making colored extracts from the treated methylene blue such as xylol, toluol, benzol, turpentine, ether and chloroform were tried. Of those investigated, the best solvent for the derivatives of methylene blue was chloroform, which took twenty per cent. of the 105-minute purple specimen. Toluol was next best, ether third, and carbon bisulphide fourth. It was noted that when a chloroform extract was first made, no further extraction could be made with either toluol or ether. Utilizing these findings, four successive extracts (first, ether; second, toluol; third, chloroform; and lastly, carbon bisulphide) were made of a specimen containing one per cent. methylene blue, and 0.5 per cent. sodium bicarbonate, boiled for ten minutes. Similar extracts were made from the same substance. boiled 105 minutes, and the increased color in each extract compared by colorimetric measurements with the corresponding one of the first series. Care was taken in the second series to make the

last washing of each extraction below the standard color before the next solvent was employed. The colored material in the ether extract increased ninety times, that in toluol, fifty-two times, chloroform, ten times, and carbon bisulphide, three times.

It is difficult to explain these results except on the assumption that there were present in certain extracts at least four color-producing compounds, and that they are formed in different ratios by the above methods. The results harmonize with the further fact that the dried extracts when dissolved in boiling water, formed four different crystals, together with considerable amorphous material.

Probable Formation of Thionin or Thionol.

In another experiment, it was noted that very little matter soluble in ether and chloroform was present in the filtrate of a methylene blue specimen boiled for eight hours with an excess of silver oxide. The substance in the residue was red and might be supposed to be the free base of methylene blue, which gives a reddish color and is insoluble in ether and chloroform, but its acidification with hydrochloric acid and the subsequent addition of alcohol gave a brown ring with sulphuric acid, not a green one, as would be the case, had the substance been the free base. On further examination the eight-hour filtrate gave some reactions very similar to thionol, among these being its ability of uniting with eosin and of forming a metallic purplish precipitate. This fluffy product could not be the eosinate of methylene blue, since the latter is dark blue, not purple. It could not be methylene violet, since methylene violet remains along with the original precipitate in its unaltered crystalline form. The filtrate from it contains almost as much material soluble in ether [29] as does the original methylene blue mixture untreated by eosin, and therefore it could not be methylene azure. This purple compound of eosin was soluble in methylated spirits, gave staining reactions very much like those resulting when thionin was similarly treated with eosin, and was, therefore, considered to be thionin or thionol.

[29] Ether will not dissolve eosin, methylene violet, or the eosinates of methylene blue, thionol, or its closely allied bodies.

SOME CONSIDERATIONS REGARDING THE MAKING OF A METHYLENE BLUE-EOSIN STAIN.

From the above, it is evident that the derivatives of methylene blue essential for a good stain may be different in their composition and varied in their mode of production. A very fine stain is secured by boiling for twenty minutes a quantity of one per cent. methylene blue, containing 0.5 per cent. sodium bicarbonate, and at least 0.5 per cent. freshly precipitated silver oxide. One-third is then removed, and after another twenty minutes, half of the remainder is withdrawn. The process is continued with the rest of the mixture for the remainder of the hour. The three products are reunited, distilled water added to compensate for the loss by evaporation, and the mixture allowed sufficient time for the precipitate to settle. The silver oxide in the above process being 0.03 per cent. soluble in water, and ionizing into Ag and OH, reacts on the chloride of methylene blue, forming the free base, according to the equation $Mb — Cl + Ag — O — H = Mb — O — H + AgCl$.

Depending on the chemical nature of derivatives of methylene blue, the action of sodium bicarbonate may be one of oxidation or of reduction. The $Mb — O — H$ in the presence of these reagents readily forms derivatives, among which are methylene azure and methylene violet, the former being an oxidation process, and the latter, a reduction process. The reduced body, possibly thionin, or its highly oxidized body, thionol, might be formed partly by the interaction of other derivatives. One-half per cent. filtered eosin was next added to an equal volume of the prepared mixture. Theoretically, the weights of the untreated dyes should be taken in the ratio of 824 to 746 or one gram of eosin to 0.92 gram of methylene blue, but on account of the free bases of methylene violet and methylene azure not uniting with eosin, the weight of the latter taken was one half that of the methylene blue. An excess of either eosin or methylene blue must be avoided, since it was found that not only the precipitates formed are soluble in the excess of either reacting body, but also all the crystalline material occurring in the treated mixture is likewise soluble. The reaction of the free base

of methylene blue with eosin is represented thus: $2Mb-O-H+Eo.K_2=Eo.Mb_2+2KO-H$. Whenever it is desired to form eosinates of methylene violet and methylene azure the slight alkalinity of the mixture should be reduced by almost neutralizing it with very dilute hydrochloric acid before the eosin is added. There is no good reason for diluting the reacting bodies, since the eosinates are less soluble in neutral salts than in distilled water. Moreover, the reaction occurs equally well, whether eosin is added to the mixture or the mixture to the eosin. It might appear that the dark red amorphous silver eosinate, previously referred to, might be formed as one of the products in the above manipulation. This salt, however, could not be detected microscopically after an hour's treatment of eosin with silver oxide, and .05 per cent. sodium bicarbonate.

The Use of Methylated Spirits as a Solvent.

The eosin combinations and other precipitates which may be mingled with them, after standing for an hour or so, are thrice filtered and washed with distilled water or preferably by a solution of sodium chloride to remove any free alkalies which in subsequent staining would injure the tissues and likewise give great prominence to the basic part of the stain. Methylated spirits has an economic advantage over pure methyl alcohol, and was found better adapted for the staining technique which was employed. It was, therefore, used in dissolving the precipitate above described, as it had been previously employed in studying the staining reactions of the other eosin compounds, including those of thionin, thionolin and thionol. The stain was made half saturated by adding the filter and its contents to the alcohol and bottling the same for several hours. The saturated solution thus formed was filtered and to the filtrate was added an equal volume of methylated spirits.

In this stain there are present at least seven compounds, the eosinate of methylene blue, a small amount of methylene azure, considerable methylene violet, the probable eosinates of thionin, thionolin, and thionol, and, according to Rosin, some methylene orange, and a black precipitate, insoluble in ether.

Technique and Staining Results.

Modified methods were employed in making smears for testing the above stain. Strips of tissue or cigarette paper having a width of the cover glass were cut, and in work with undiluted blood, one edge slightly moistened with physiological salt solution. Over this moist edge were passed two cubic millimeters of blood, measured from a medicine dropper of very fine bore. The strip containing blood was then quickly drawn across the cover glass at an angle of 60° with the slip. The smear was quickly air-dried, and was then thrice passed through the flame.

The manner of using the stain is simple. Add a few drops to the specimen; wait until a scum forms on the surface (its appearance depending upon the amount of the stain used), after which a few more drops are momentarily poured on the smear, so as to dissolve any precipitate that may have formed among the corpuscles. Wash with distilled water; dry thoroughly and mount in thick Canada balsam. In staining the malaria parasite especially, thin balsam should be avoided, as the xylol in it dissolves out the methylene azure and methylene violet, and thus mars the permanency of the preparation.

The staining as above described showed red corpuscles colored terra cotta to pink, the granules of the polymorphous neutrophiles pink, of the eosinophiles red, those of the basophiles purple, the bacteria dark blue and the nuclei of the polymorphous cells pale blue. the lymphocytes deep blue with a halo of pale red sometimes containing faint purple granules, and the platelets pink, while there was a total absence of the amorphous precipitates so frequently marring blood stains. A deeper blue staining effect may be secured when desired by adding to the stain one per cent. methylene blue dissolved in methylated spirits previously freed from acids by shaking with calcium hydrate.

Where the stain is desired for detecting malaria parasites, the dried red ether extract from a thirty-minute specimen can be dissolved in methylated spirits and added to the original stain.

SOME THEORETICAL CONSIDERATIONS IN STAINING.

It was noted that the extracts of the mixtures in ether, xylol, benzol, toluol, chloroform, and acetone were always red. This color seemed due to the power these solvents had of taking up red compounds in their unaltered form. This view was sustained by the mixtures becoming less red and more bluish after the extracts were made. In water, the dried red ether extract is very slightly soluble with a blue color which persists in an atmosphere of hydrogen or of carbon dioxide. From what Heidenhain[80] has found regarding Nile blue base, we should suspect that the carbon dioxide of the water had united with the base and formed the blue colored salt, when the extract had been evaporated in the air. This suspicion proved to be correct, since a considerable quantity of the dried extract gave bubbles of gas when treated with hydrochloric acid. When the same extract was dissolved in alcohol, previously treated with calcium hydrate, the liquid had a reddish tinge. The same extract suspended in water, treated with dilute sodium hydrate gave microscopically a reddish precipitate which, when redissolved, in ether produced a fiery red color. All these facts point to the base or bases as being red, and that the six solvents above mentioned dissolve them unchanged. In water and in alcohol the products become bluish or purplish, because salts were formed from the base uniting with the carbon dioxide of the atmosphere on the one hand, or some acid body in the alcohol on the other. On theoretical grounds, the color produced could not be due to the formation of different ions, since the colored ions of the free base occurring in treated methylene blue, would still remain the positive colored ions in the newly formed salts, while the negative ions of these salts would be colorless. From this the conclusion can be drawn that the blue color must be due to the color of undissociated salts.

The staining effects already described in this paper require some explanation. It was mentioned that the red corpuscles were sometimes stained green or blue, sometimes red or brown. Where the

[80] Heidenhain, *Pflüger's Archiv f. d. gesammte Physiologie*, 1903, c, 217.

corpuscles were bluish, the base of the methylene blue derivative united with an acid radical of the cell proteids and formed a blue or green colored salt. Where a red color appeared, the acid radical of eosin had united with the basic radical of the proteid and formed a blue colored salt. The nuclei always contained nucleic acid and hence were always able to form the blue or purplish salts with methylene blue or its derivatives. Even the stain itself, was of a violet hue, as a summation of the eosin and methylene blue colored chromogens.

It was noted that the staining of a specimen occurred slowly when the evaporation of the alcohol was prevented, but when the alcohol was freely allowed to volatilize, the characteristic colors developed quickly. A couple of tentative experiments were made on the conductivity of the stain, diluted with distilled water, with a view to possible explanation of this and certain other phenomena. The conductivities of the solutions examined expressed in reciprocal ohms at 5° C. multiplied by 10^8 are here given for two different experiments.

First Example.

Undiluted stain	1.58
Distilled water	0.28
Equal parts of stain and distilled water	0.44
Two parts of stain and one part distilled water	1.07

Second Example.

Undiluted stain	1.85
Distilled water	0.37
Equal parts of stain and distilled water	0.62

The gram equivalent in terms of sodium chloride was found to be .0018, as calculated from my chart,[31] and hence, would require the same gram equivalent of the completely ionized eosinate of methylene blue to give the same conductivity. This latter value would correspond to 0.28 per cent. of the compound dye, and is much above the medium, since a large portion of the conductivity of the stain must be due to inorganic ions. Instead of the conductivity of a mixture of equal parts of the stain and water being the average between the water and the stain, when separately examined, its value in the above examples was only about half this

[31] Wilson, *Amer. Jour. of Physiol.*, 1906, xvi, 447.

average. The change in the conductivities of the stain was found to be due to the formation of a microscopic precipitate.

The addition of water to the stain is comparable to the loss of alcohol by the evaporation which occurs in the method of staining above described. The residue in the latter case becomes more and more aqueous as the volatilization proceeds. In both processes there is a precipitation of the dye and a reduction in the number of ions. The more rapid staining when free evaporation is permitted may be due to the dye molecules or ions being more rapidly brought within the absorption sphere (0.05 μ according to Quincke), or within the chemical sphere of the cell elements concerned.

The staining effects in either case cannot be explained according to the physical or solution theory of Witt and Michaelis[32] since methylene violet and methylene azure are more soluble in methylated spirits than in any of the solvents studied. It seems well-nigh impossible that the dye molecules or ions should be relatively so soluble in the cytoplasm and in the nuclei of the cells as to enable the stain to be so condensed in these cells, as certainly occurs in the staining act. In order to realize the truth of the latter statement, one has only to examine the stain microscopically to see how weak in color it is as compared with the same thickness of stained material. Moreover, the stain is largely colloidal, or at least diphasic[33] and, therefore, diffusion would be too slow to produce the staining effect in the short time ordinarily required for the staining act.

SUMMARY.

1. Eosinate of thionin gives very satisfactory staining for blood smears. It is easily prepared and dissolves readily in methylated spirits.

2. In the methods heretofore employed for making mixtures of eosin and methylene blue derivatives, the eosinates of methylene violet and methylene azure are present in very small quantities or are altogether absent.

[32] Michaelis, *Pflüger's Archiv*, 1903, xcvii, 634.

[33] Barratt, *British Bio-Chem. Jour.*, 1906, i, 424.

3. Thionol and thionin are probably formed in methylene blue which has been long boiled with dilute alkalies and silver oxide.

4. Good stains of eosin and methylene blue derivatives can be obtained by a variety of manipulations.

5. Methylated spirits is more economical as a solvent for this stain and better adapted for the simple technique above described than is methyl alcohol. There is some evidence that the staining act is of a chemical nature.

FEVER: ITS METABOLIC CHANGES.[1]

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Krehl and his pupils have stated that the material which is oxidized in the fever generated by puncture of the thermogenic center is different from that oxidized in infectious fevers. In neurogenic fever (puncture of thermogenic centers) they infer that the fever is due to an increased metabolism of glycogen, while the increased metabolism of proteids in it is due not to the puncture but to the high temperature. In infectious fever, on the contrary, there is a toxic metabolism of the proteids, since the excitant of fever acts upon all the tissues of the body and in that way increases the metabolism of proteids. Hirsch and Rolly have shown in the neurogenic fever and the infectious fever that the liver has the highest temperature. Hirsch and Rolly have shown that in curarized animals puncture of the nerve centers causes an elevation of the temperature.

Manassein found that in fever and in hunger the amount of glycogen present was considerably diminished and finally completely vanished.

Rolly determined the diminution of glycogen in the liver and in the muscles in neurogenic fever and in the fever of infection. Rolly found the amount of glycogen diminished in toxic and in neurogenic fever, in strong muscular work, and in fasting of the animal.

Hirsch and Müller have shown that as the liver has the highest temperature, in the liver must be found the greater metabolic changes. The question then arose, whether after puncture of the thermogenic centers in animals freed of glycogen there ensued an

[1] Received for publication September 19, 1907.

elevation of temperature. Rolly found that in twenty-one rabbits with no glycogen in the muscles or liver, puncture of the thermogenic center did not cause an elevation of temperature. In two rabbits only was there a rise of 0.4 and of 0.2° Fahrenheit. To animals free of glycogen he fed simple syrup to produce glycogen again; puncture of the brain then produced fever. In glycogen-free animals the injection of bacteria (pneumococcus and bacterium coli) produced a fever; hence in glycogen-free animals infection generates fever, but in the same animal thermogenic puncture does not.

Rolly also found that albumoses and peptones do not generate fever in glycogen-free animals. One of us long ago[2] proved that they generated fever in animals in normal condition.

Rolly also found that there was no increase (or only a small one) of urinary nitrogen after thermogenic puncture in glycogen-free animals. Hence Rolly supports Krehl and Schultz in their theory that the small increase of urinary nitrogen after brain puncture is due to the hyperthermia and is not a direct result of the puncture of the thermogenic center. In the glycogen-free animals not only does the thermogenic puncture produce no fever, but the increase of urinary nitrogen does not take place to any extent. Rolly believes that in neurogenic fever the increase of urinary nitrogen is due to the hyperthermia and not to the irritation of the thermogenic nerves. The greater increase of urinary nitrogen in fever generated by bacteria is due to an increased destruction of proteid, produced by the infection itself. In infectious fever there is from the beginning an abnormal destruction of proteid.

Hirsch, Müller and Rolly hold to the theory that in fever we have two parallel processes: (1) a specific breaking up of the proteid by the bacteria, and (2) a central excitation in the sense of a neurogenic fever.

Aronsohn has opposed this view. He believes that the increased destruction of proteid is dependent upon the nerves and ferments. The theory of a toxic destruction of proteid is without foundation. An increased destruction of proteid ensues according to him (1) where there is a paucity of glycogen and fats; (2) in toxic fever

[2] *Jour. of Physiol.*, 1887, viii, 218.

and in excessive irritation of the nerves, and (3) in cachexias. The increased destruction of proteid for Aronsohn is a result of the fever-process due to heightened innervation of the cells—an irritation of a thermogenic center.

It occurred to us to study the effect of an agent, beta-tetra-hydronaphthylamin, upon glycogen-free animals. This body is a pure nervous agent in the production of fever. One of us has in another place[3] shown that it acts only when the corpus striatum and tuber cinereum are present. If only the corpora striata are removed, still the irritations of the thermogenic centers in the tuber are sufficient to produce a fever. The first experiment shows the effect of beta-tetra-hydro-naphthylamin in normal rabbits. The second experiment shows that there is a rise after a transverse section of the brain behind the corpora striata. The third experiment shows that there is no rise after section behind the tuber cinereum.

Experiment 1.—Black rabbit.

Time.	Temperature.	
2:45 P. M.	102.4° F.	
2:50		.05 gram of naphthylamin per jugular.
2:55	103.1	
3:00	103.4	
3:10	104.0	
3:25	104.3	
3:35	104.8	
3:40	105.0	
3:55	105.6	
4:10	106.2	
4:30	106.8	
4:40	107.1	
4:50	107.3	+4.9° F.

Experiment 2.—Rabbit was etherized and jugular prepared; brain exposed.

4:30 P. M.	101.8° F.	Transverse section on a line behind the corpora striata.
4:31		½ drop of beta-tetra-hydro-naphthylamin per jugular.
4:35	102.2	
4:43	102.5	
4:46	102.8	
4:51	102.9	+1.1° F.

[3] *Med. Bull.*, 1898, xx, 411.

Experiment 3.—Rabbit; transverse section through the tuber cinereum.

2:20 P. M.	Before section.	101.8° F.	
2:35	After section.	101.0	.005 gram of naphthylamin injected per jugular.
2:40		101.1	
2:45		101.2	
2:55		101.2	.055 gram of naphthylamin.
3:00		100.6	
3:05		100.6	.010 gram of naphthylamin.
3:30		100.2	
3:50		100.2	
4:00		100.1	
4:05		100.1	

We next proceeded to make rabbits free of glycogen. The animals received water but no food for four to five days. They then received subcutaneously one cubic centimeter of a .01 per cent. solution of sulphate of strychnia at intervals of fifteen minutes, until spontaneous convulsions ensued. These convulsions were kept up by sensory irritation and injections of strychnia. If the convulsions could not be provoked another injection of strychnia was made. This was kept up about four hours. They were then permitted to rest for a while, when they received intra-muscular injections of adrenalin. In some cases intra-muscular injections of adrenalin were made before the use of strychnia. After a rest of about twenty hours they received hypodermic injections of naphthylamin. when the temperature was noted. To determine the amount of glycogen present we proceeded as follows: The animal was killed by a blow on the neck. The liver was removed, weighed and quickly thrown into a boiling (1.7 per cent) solution of potash. The boiling was kept up for about a half hour, until the pieces of liver were dissolved. A definite quantity of this alkaline solution was neutralized with hydrochloric acid, and the hydrochloric acid and iodide of mercury added until no more precipitate ensued. This solution was filtered and the filter, with the precipitate, dissolved with fifteen per cent. solution of potash and water and again precipitated with hydrochloric acid and iodide of mercury. This precipitate was again dissolved in potash solution and water and

the solution again treated with hydrochloric acid and iodide of mercury. All the filtrates were then put together and precipitated with two volumes of 96 per cent. alcohol. The glycogen now separated in flakes and fell to the bottom. This solution was again filtered and the glycogen on the filter washed with alcohol and ether, and then dried.

The muscles of one half of the body after fine division were treated in the same manner. In some cases the liver and muscles were combined and treated by the above method.

Senator and Richter have calculated that a normal rabbit, with its heat economy remaining the same and of a weight of two kilograms, needs 60 calories per kilogram or 120 calories to maintain its temperature. If the normal temperature of the rabbit is 39° C. and the air temperature 19° C., then to keep the temperature normal 120 calories will be necessary, an elevation of temperature of 39° C. less 19° C. equals 20° C. For a rise of one degree centigrade in the rabbit there would be needed six calories (120/20 = 6 calories), to elevate the temperature of the rabbit 2° C., 12 calories. One gram of glycogen by combustion yields 4.1 calories, so that three grams of glycogen would have to be oxidized to raise the temperature two degrees centigrade. The presence in the rabbit of .01 gram of glycogen would not affect the conclusion that a rise of two degrees Fahrenheit in the rabbit cannot be ascribed to the glycogen. The following experiments show the rise of temperature in animals deprived of glycogen:

Experiment 4.—Gray female rabbit was given cabbage diet for five days; no food for four days except water.

7/16/'07.

7:00 P. M.	Adrenalin 15 mgr. by intra-muscular injection.	
10:00 A. M.	Hypodermic injection 1 c.c. strych. sulph. (.01 per cent.).	
10:15	Same injection.	
10:30	" "	
10:45	" "	
11:00	" "	
11:15	" "	Convulsions.
11:30	" "	
11:45	" "	
12:00	" "	

12:30 P. M.	Same injection.
1:00	" "
2:00	" "
3:00	Injection of .5 c.c. strychnine sulphate solution.
4:00	Same injection.
5:00	" "
6:00	" "
7:00	" "

Convulsions continued until 9 P. M.

10:00 P. M. 15 mgr. adrenalin intra-muscular injection.
7/17/'07.
7:00 A. M. 15 mgr. adrenalin intra-muscular injection.

Time.	Temperature.	
3:00 P. M.	100.5° F.	.01 gram naphthylamin hypodermically.
3:10	100.6	
3:20	100.8	.01 gram naphthylamin hypodermically.
3:30	101.0	
3:40	101.2	
3:50	101.4	
4:00	101.6	
4:10	101.8	
4:20	102.1	
4:30	102.4	
4:40	102.6	
4:50	102.7	
5:00	102.8	
5:10	102.9	
5:20	103.0	
5:30	103.0	+2.5° F.

Liver, weight .... 46.0 grams.	Precipitate and filter	1.42 grams.
	Filter	1.41
	Glycogen in liver	.01
Muscles (leg, arm and side), weight .... 73.0 grams.	Precipitate and filter	1.39 grams
	Filter	1.39
		.00

No glycogen was found in the muscles.

Experiment 5.—Black female rabbit was given cabbage diet five days; no food for four days except water.

7:00 A. M. Adrenalin 15 mgr. intra-muscularly.
1:00 P. M. Injection of 1 c.c. strych. sulph. sol. (.01 per cent.).

1:15	Same injection.	
1:30	" "	
1:45	" "	Twitchings.
2:00	" "	
2:15	" "	
2:30	" "	
2:45	" "	Spontaneous convulsions until 11:00 P. M.
3:00	" "	
3:30	" "	
4:00	" "	
4:30	" "	
5:30	Injection of 0.5 c.c. strych. sulph. sol.	
6:30	Same injection.	
7:30	" "	
8:30	" "	
11:00	Adrenalin 15 mgr. intra-muscularly.	
7/23/'07.		
7:00 A. M.	" " "	
12:00	" " "	

Time.	Temperature.	
4:00 P. M.	102.4° F.	.01 gram naphthylamin hypodermically.
4:10	102.5	
4:20	102.6	.01 gram naphthylamin hypodermically.
4:30	102.8	
4:40	103.0	
4:50	103.2	
5:00	103.5	
5:10	103.7	
5:20	103.9	
5:30	104.1	
5:40	104.2	
5:50	104.3	
6:00	104.4	
6:10	104.5	
6:20	104.5	+2.1° F.

Liver, weight	43.0 grams.	Precipitate and filter	1.31 grams.
		Filter	1.30
		Liver glycogen	.01
Muscles (leg, arm and side), weight	92.0 grams.	Precipitate and filter	1.27 grams.
		Filter	1.27
			.00

No glycogen was found in the muscles.

Experiment 6.—Brown rabbit was given cabbage diet for five days; no food for four days except water.

7:00 A.
10:00
10:15
10:30
10:45
11:00
11:15
11:30
11:45
12:00
12:30 P.
1:00
1:30
2:30
3:00
3:30
4:30
5:30
8:00
7/10/'07.
8:00 A.

Time.
1:00 P. M.
1:10
1:20
1:30
1:40
1:50
2:00
2:10
2:20
2:30
2:40
2:50
3:00
3:10
3:20
3:30
3:40

Liver, weigh
Muscles (le
side), wei

Experiment 7.—Black rabbit was given cabbage diet for five days; no food for four days except water.

7:00 A. M.	Adrenalin 15 mgr.
12:00	Hypodermically 1 c.c. strych. sulph. sol. (.01 per cent.).
12:15 P. M.	Same injection.
12:30	" "
12:45	" " Twitchings.
1:00	" "
1:15	" "
1:30	" "
1:45	" " Spontaneous convulsions to 9:00 P. M.
2:00	" "
2:30	" "
3:00	" "
5:00	Injection of .5 c.c. strych. sulph. sol.
6:00	Same injection.
7:00	" "
8:00	" "
9:00	" "
11:00	Adrenalin 10 mgr. hypodermically.
7/10/'07.	
7:00 A. M.	" 15 " "

Time.	Temperature.	
2:25 P. M.	102.8° F.	.01 gram naphthylamin hypodermically.
2:35	103.0	
2:45	103.2	.01 gram naphthylamin hypodermically.
2:55	103.6	
3:05	104.0	
3:15	104.2	
3:25	104.4	
3:35	104.5	
3:45	104.7	
3:55	104.8	
4:05	105.0	
4:15	105.1	
4:25	105.1	
4:35	105.1	+2.3° F.

Liver, weight	56.0 grams.	Precipitate and filter	1.33 grams.
Muscles (leg, arm and side), weight	98.5 grams.	Filter	1.33
	154.5		.00

We performed twenty experiments. We did not find it an easy matter to free the animals completely of glycogen; but we had

some completely freed of it. We have given some examples of our results. They showed that beta-tetra-hydro-naphthylamin will produce fever in a glycogen-free animal. The fever here must be due to a using up of the proteid. The metabolism of the proteid is set into activity by the stimulation of the thermogenic centers in the corpus striatum and the tuber cinereum, for the removal of these centers prevents the naphthylamin from causing a rise of temperature.

That puncture will not cause any rise of temperature in a rabbit free of glycogen is probably due to a weak stimulation of the thermogenic centers. Naphthylamin is a much more powerful stimulant and is like the poisons of infectious fevers. Albumoses and peptones are also probably too weak as stimulants to the thermogenic centers in a glycogen-free rabbit. Here the naphthylamin stimulates the nerve centers, which cannot act on glycogen, but acts upon the proteid initiating changes in it. These facts do not support the views of Krehl and Rolly that puncture of the brain acts only on glycogen, while the infectious fevers produce a toxic metabolism of proteid.

Nearly all observers agree that in fever there is an increased proteid metabolism, but no increased fat metabolism except such as may result from inanition in the individual. There is every reason to believe that with both puncture of the thermogenic centers and with the infectious fevers, fever is produced by an action on the thermogenic centers. As Aronsohn has contended, there is no toxic destruction of proteid except through the trophic nerves of the thermogenic centers. The intra-cellular ferments also have a share in the metabolic changes of fever.

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INDEX TO VOLUME IX.